

Parliament's chance to act

This week's debate in the House of Commons about the British government's handling of research and the universities could be a turning point, but the critics must take careful aim.

THE research enterprise and the university system with which it is linked have been badly battered in the past six years, in part because they lost their political friends a long time ago. That is also why it is a long time since the condition of British science was last debated in the House of Commons. (The House of Lords, more leisured but less influential, has been more attentive.) Labour, the chief opposition party, has been crying out for months for a debate about Sir Keith Joseph's part of the enterprise, the universities and the research councils, but has not gone so far as to sacrifice some of its own ration of parliamentary time. Now the government has arranged a debate for Friday, when the House of Commons will be almost empty. Complaints will be aired in a near-vacuum. Let us hope they do not go unheard.

As always, it is important that the complaint should not be overstated. Complainants should at the outset concede a large part of the government's defence. There have been damaging budget cuts, but the Prime Minister has kept her promise, in 1980, to "protect" the direct income of the research councils, the continuing need to reduce public spending notwithstanding. The cuts have fallen instead on the universities in a way that has damaged the underpinning of research, and on the research councils indirectly, by the whittling away of what government departments can afford to spend on commissioned research. The latest cut, by £10 million this year and £20 million next year, in the budget for basic agricultural research, has arisen from the separate dealings between the Treasury and the agriculture ministries, but in a way that suggests the machine is out of control.

Complainants must also concede that there is no reason why research and the universities should have been exempt from the financial pressures to which other government spending has been subject. Most universities are no doubt less efficient than they might be. The research councils are not uniformly paragons of good management (and some of them need urgently to be reorganized). There may even be something in the opinion that because British spending on research and higher education has grown as a result of ill-coordinated decisions to an arbitrary level, it may now be arbitrarily cut back. Put simply, the case against the budget cuts is not that they have been made, but that they have been decided and enforced without much thought for the consequences. Cash limits have become a substitute for thoughtful policy. Paradoxically, the results include not only economies but also diseconomies.

Collapse

The collapse of the dual-support system is the best known diseconomy. Britain has boasted of this device for supporting university research bimodally, by means of project grants from the research councils to researchers whose competence is assured from the recurrent university budget, which provides basic equipment, technician help and other elements of infrastructure. The system has been under strain for at least a decade, as universities have spent their shrinking funds on survival rather than research support. For at least five years, only the strongest universities have been able to support research groups adequately, often by spending precious reserves or by running up debts.

One result is that research council funds intended as research grants must be spent instead on basic research support. Another,

more damaging but more common, is that able research groups are frequently ill-provided with research support, even in competitive fields where being second counts for very little. In due course, this imbalance will be put right as the University Grants Committee implements its declared policy of transferring funds within away from less active university departments and even entire universities. But it will be a decade before that policy takes effect. One consequence will be a reduced demand for public research funds, another will be a reduction of the volume of publicly supported research, a third that many creative people will be stranded in places deemed unworthy of public provision of research support. No wonder that people no longer whistle on the way to their laboratories.

The present system is often described as wasteful by its critics, but the remedy now prescribed entails waste not previously imagined. The few thousand researchers from universities and research establishments put out to grass in the past five years have been persuaded to "take" early retirement by financial inducements whose immediate cost (known as that of "restructuring") must be close on £50 million, but whose real cost will be buried in pensions plans for years to come. But the much more serious cost may be that implied by the frequent practice, among those displaced, of working profitably for employers outside Britain. That little irony, with all its implications, deserves a mention on Friday. So, no doubt, will be the diseconomies enforced by the common need to operate educational plant below capacity (because of constraints on student numbers) and to let research equipment lie idle (because operating funds are not to be had). Much of the experience of the past few years is a proof of the diseconomies of contraction.

The British government's rejoinder is that many of these problems would be less serious if only universities would do what they should have been doing all along, and forging stronger relationships with industrial companies. Again, it should be conceded that many (but not all) researchers have been slow to recognize that there may be sources of support other than the government, with the advantages of plurality that would then accrue. But it is unreasonable to expect that such funds will be forthcoming on a scale sufficient to make up for present deficiencies. It would be positively undesirable, and probably an offence against the rules of spending public money, that all hard-pressed institutions should top up their budget deficits in this way. But, in any case, such industrial funds as there may be will probably be distributed on the Matthew principle ("to him that hath...") and will be concentrated among the better off.

Biological manuscripts

Miranda Robertson, Biological Sciences Editor of *Nature*, is now based in the Washington office of *Nature*. Manuscripts offered for publication may be sent, as at present, to either the London or the Washington office; with the benefit of good communications, the speed with which manuscripts are dealt will be independent of the place at which they are received. But authors are asked please in future to send **four copies of manuscripts** intended for publication (one for each office and two for referees).



Closer industrial liaisons will, however, make for a more effective transfer of science and technology from research laboratories to British industry. That has been a constant part of the government's recent opinions. The argument has some force, but not all industrial liaisons with research are desirable or can even be expected to lead to worthwhile commercial enterprises. There are many fields of research (software development, for example) in which it is more important that universities and public laboratories should remain impartial sources of advice for a whole sector of industry. And the ragbag of innovations the universities are likely to produce under the new arrangements for their exploitation will not amount to a new industrial pattern for Britain, however valuable some of them may be. There is nothing wrong with the government's wish that British civil science should be more directly productive (than by the provision of manpower) of prosperity, but the naivety of the assumptions often implied about the nature of major industrial innovation suggest that some enterprising university might profitably design an elementary course in economic history for the British cabinet.

Part of the British government's difficulty in this connection is that it lacks machinery for considering these issues in the round. (The Advisory Committee for Applied Research and Development is a group of part-timers which seems to have gone to sleep.) But there are important issues crying out for remedy beyond the immediate boundaries of the Department of Education and Science. The steady erosion of the research budgets of the other civil ministries (other than the Department of Trade and Industry) suggests the government has abandoned the inconvenient Rothschild principle that all ministries should be competent to pursue research. Is it wise that such a state of affairs should come about by neglect?

The problems at the Ministry of Defence are the opposite: research and development costs not much less than the whole of what the government spends on civil science, and is growing. Yet it is now nearly two years since the Prime Minister and the Secretary of State for Defence promised in public that defence research would be made more accessible to British industry. Ministers are busy people, so that it is understandable that such tasks, full of bureaucratic minefields, should be set aside. What puzzles and demoralizes those who work in civil research is the zeal with which change has been ordained in the universities and the research councils.

Morale

Morale, not money, is what matters in the end. The dictum that the management of success in research requires the creation of an intellectual climate in which good ideas (and their authors) can prosper is none the less true for being over-used. And although research managers (in universities as well as public laboratories) could have done more to protect creative people from the corrosive insecurity that afflicts them, these same people read the newspapers and learn what government spokesmen have been saying about the value of their work. Sir Keith Joseph's own white paper on higher education (see *Nature* 23 May, p.270) is the most striking recent example of the genre. By dodging questions whose answers might have resolved uncertainty, by its view of higher education and research as an input/output system and by its repetition of a naive view of where industrial innovation comes from, it can only have made British academic researchers think the time had come to go elsewhere. It seems a pity to hazard the productiveness of a whole community for modest savings at its margin.

The British government's weakness is that few of the changes now decreed, many of them well-intentioned, have been carefully thought through. If the proper calculations had been made, change would have been more deliberate, not necessarily slower. And even at this late stage, much could be done to put things right. Making the system more coherent, perhaps by appointing a Minister of Science (part-time) would be the best course. Taking careful stock in the hope of learning where the research enterprise is heading would be worthwhile. Replacing the policy of management by cash limits with something more intelligent is

essential. And, for the time being, the issue is not one of party politics: with the general election in Britain two years or so away, the erosion of morale is too rapid to allow an opposition the conceit that it might be possible to wait until then to earn the kudos of reform, for there may be little left by then. □

The tax man cometh

President Reagan's recent reforms go only part of the way towards fairness.

FORMER President Richard Nixon used to speak of various of his administration's actions as "the greatest (fill in the blank) since the Creation"; President Ronald Reagan may thus be forgiven for calling his tax reform proposal merely "the Second American Revolution".

Amid the hundreds of pages of simplification in Reagan's plan is the retention of the research and development tax credit, a provision that, according to independent economists, has cost the Treasury \$1,000 million a year to stimulate \$700 million worth of research and development. High technology and biotechnology companies, while grumbling along with other industries about the net 25 per cent increase in corporate taxes embodied in the Reagan plan, are full of praise for the wisdom of keeping the research and development tax credit. Sentiment in Congress is mixed, and since it seems unlikely that the Reagan proposal will be disposed of this year, and since the research and development credit is due to expire this year, it will take a special action by Congress to retain it.

The tax credit was passed under a banner of stimulating the United States' supposedly lagging investment in the research and development that tomorrow's products will depend upon. Research and development costs had, of course, always been deductible as an ordinary business expense; the tax credit provides an extra bonus — an outright rebate of taxes equal to 25 per cent of a company's research and development spending that exceeds its average outlay in this area of the preceding three years.

Yet, as recent studies have shown, the credit has probably done little to stimulate new research. In fact, the credit has in effect been a reward for those companies that had already decided to invest in research and development. To those who know the politics that were at work in passing the enabling legislation four years ago, that is probably no surprise.

Of course, the sponsors of the legislation did not come out and say so. But the authors of the legislation will now admit privately that the true purpose of the bill was to equalize what the high-technology companies saw as unfair treatment in the tax code. Heavy manufacturers were reaping a windfall through the investment tax credit (which gives a tax rebate of 6-10 per cent on new purchases of machinery) and accelerated depreciation. So lucrative have these provisions been that many leading US manufacturers have paid no taxes for years. The high technology companies, whose products depend more on research and development than machines, did less well. Thus was born the research and development tax credit.

If tax simplification is truly the President's goal, then such backhandedness has no place in the tax code. The whole story of special interest politics has been one of obscure provisions masquerading as lofty general principles but in fact carefully crafted to benefit group X (sometimes company X) that gave a nice campaign contribution to the right congressman. And the whole principle of tax simplification is the elimination of special treatment for some in favour of lower rates for all.

The administration is proposing to bite the bullet and eliminate the over-favourable treatment of capital investment: under the Reagan proposal, both the investment credit and accelerated depreciation would be repealed. And a new minimum corporate tax would put a further effective cap on the benefits that the heavy manufacturers can reap from capital investment. The cause of simplification and fairness might further have been served by going further with the minimum tax, and eliminating the research and development credit. □

US medical research

Hughes tops league of US charities

Washington

THE Hughes Medical Institute became the world's wealthiest charity last week when it sold off Hughes Aircraft Company to General Motors for an estimated \$5,000 million. Dr Donald Frederickson, president of the institute and former director of the National Institutes of Health (NIH), said the new endowment should allow the institute to more than double the \$100 million it currently spends each year on medical research in genetics, immunology, neurosciences and metabolic control.

The sale also marks a further step in the demystification of the institute, which has been something of an enigma even to the scientists it supports. There is no such thing as applying to the Hughes Institute for a research grant; it only gives money to "affiliate" laboratories, of which there are now eighteen, on medical school campuses around the United States. The institute pays the salaries of the 200 or so scientists who work in these laboratories and leases the buildings from the universities. The most recent campus to join the select ranks is the University of California, San Diego and the Salk Institute. Others include Harvard, Yale, Johns Hopkins and Stanford Universities.

Like its mysterious founder, the institute has tended to operate beyond the public view. It has often been criticized for failing to maintain any open criteria (including peer review) for selecting researchers and projects to support.

That has changed dramatically, however, since Frederickson took charge in 1983, following a court fight in Delaware (where the institute is legally incorporated). In the same year, Delaware's attorney general succeeded in removing most of Hughes's former associates from the board of trustees.

The Hughes Institute's \$5,000 million places it far ahead of any other charitable organization or foundation. The Ford Foundation, its closest competitor, has an endowment of \$3,500 million. The Battelle Memorial Institute, which had previously been the largest independent research institute, has assets of only \$250 million.

Frederickson estimates that total public and private support of basic biomedical research totals \$1,500-2,000 million in the United States; the Hughes Institute will thus be managing some 10-20 per cent of that total. By comparison, the budget of NIH is to be \$5,200 million next year, though much of that is for clinical and applied research.

Frederickson expects the eighteen existing affiliate programmes to be expanded, as will a new scheme that he initiated to encourage medical students to consider

research careers. Under this scheme, to which the institute has contributed \$10 million, students can spend one year working at NIH with senior researchers. The 30 Hughes Research Scholars will live and work in the former Sisters of the Visitation Convent adjacent to the NIH campus; the convent is being renovated with the Hughes Institute funds.

Frederickson said the trustees are in the process of deciding whether the institute should continue business as a "medical research organization" — which, under federal tax laws, means it must conduct the research itself — or instead become a private foundation, which would allow it to make grants and expand its horizons to include medical ethics, scientific communication and science education. Medical re-

search organizations are required by law to spend 3.5 per cent of their assets each year exclusively on "continuous conduct of medical research". For foundations the figure is 5 per cent and they must also pay an excise tax of 1-2 per cent on income. In addition, foundations are subject to restrictions on their business activities.

The sale of the Hughes Aircraft Company has also removed the institute's lingering tax problems. Hughes set up the institute in 1953 as a non-profit organization which, on paper, owned Hughes Aircraft — the piece of his industrial empire that now builds defence electronics but no aircraft. For many years the Internal Revenue Service contended that the institute was being used as a tax shelter by Hughes, noting that very little of the Hughes Aircraft profits actually reached the institute. Before 1976, when Hughes died, the institute had never received more than \$4.2 million in any one year from the company. Although it was eventually acknowledged that the institute was legitimately engaged in conducting medical research, other tax problems have remained.

Stephen Budiansky

Japan in space

Carp to loop the loop?

Tokyo

JAPAN's first astronaut, who may be a woman, is scheduled to blast off in the US space shuttle in early 1988 with a chicken egg, two carp, some soyabean seeds and 20,000 fruit-fly larvae stored in Japan's section of the Spacelab Module. Not intended for eating, these organisms are vital components in a series of life science experiments designed to shed light on the effects of space travel on man.

Details of the fruit-fly experiment were revealed at a symposium held in Tokyo last week. The fruit fly *Drosophila*, which has been subject to more than its fair share of radiation on Earth, will be used to assess the possible effects on human beings of long-term exposure to space radiation, including X-rays, low-energy protons and neutrons and high-energy cosmic radiation. The larvae will be carried in an incubator aboard the spaceship and returned to Earth once they have grown to become pupae. According to Professor Mitsuo Ikenaga of Kyoto University, exposure to radiation should cause curling and/or multiplication of hairs on the wings of the adult insects, thereby providing information about chromosomal changes induced by the radiation.

In a closely related experiment, soyabean seeds will be cultivated on return to Earth and mutations in the adult plants will be easily detectable from the colour of their leaves. But Taro Fujii, head of the Plant Section of the National Institute of Genetics, fears that the degree of exposure to radiation aboard the shuttle may be insufficient to produce mutations.

In a study of space motion sickness, the

behaviour and brain-wave activity of two carp in the weightlessness of space will be examined.

A decade ago aboard Skylab, fish were observed to perform strange looping movements for the first 3-4 days in space which corresponds to the duration of disorientation reported by mission specialists. In Japan's experiment, otoliths, which give the fish its sense of balance, will be removed from one of the carp and the effects of light on the fish monitored — normal carp turn their back toward a light source.

A preliminary experiment has already been carried out by Japan's Institute of Space and Astronautical Science (ISAS) in which one carp and three goldfish in a rocket-shaped capsule were dropped from a balloon at an altitude of 30,000 m. During the 20-second free fall, the carp showed promising behaviour, although the goldfish died during the ascent of the balloon. In another experiment a fertilized chicken egg will be hatched and the effects of weightlessness on the chick's bone cells examined.

The Japanese astronaut responsible for looking after these and other experiments has yet to be selected. Of 533 applicants for the job, seven remain after three stages of screening by Japan's National Space Development Agency. These seven, including one woman, Chiaki Naito, will undergo final aptitude trials at the Johnson Space Center in Houston, Texas, beginning on 20 June, and around September three will be selected, of whom one will join the space shuttle flight scheduled for 27 January 1988.

David Swinbanks

European Community research

The RACE is on at last

Brussels

ALTHOUGH prevented by the Italian delegate from formally ratifying any decisions at their meeting on 4 June, the research ministers of the ten countries of the European Economic Community (EEC) nevertheless made several political decisions. The most tangible is the decision to launch the Community's advanced telecommunications programme, RACE (Research in Advanced Communications in Europe). In addition, there were tentative decisions on an advanced informatics programme, Community participation in the European Synchrotron Radiation Facility (ESRF) and a scheme to provide more access to national facilities.

The Italian blocking tactic came in the form of an insistence by Luigi Granelli, Italian research minister and current president of the Council of Ministers, that any proposal should be dealt with as part of an overall package. By that ploy, Granelli had hoped to prise a formal decision from his nine counterparts finally agreeing to set up the Community's tritium-handling laboratory at Ispra in Italy. A decision was blocked by the United Kingdom and France, both of which have national facilities and object to providing further funds at Community level. It now seems likely, however, that the funds will be found out of the existing budget of the Joint Research Centre at Ispra and that President Bettino Craxi of Italy and President François Mitterrand of France will tie up these and other loose ends at their summit meeting in Florence.

A green light for the definition phase of RACE (see *Nature* 28 March, p.309) was given despite problems in the meeting of the Telecommunications Council on 3 June. This phase will cost six million European Currency Units (ECU) and will last 18 months. It will start in July with the construction of a European reference model for an integrated broad-band telecommunications network that will handle voice, data and video transmissions. As a result of pressure from the United Kingdom and West Germany, the funds proposed by the Commission for the next phase will be reviewed at a later date. The present proposal for the Community to cover half the cost is also to be reconsidered with a view to more industrial participation. On French insistence, the French-chaired European Conference of Post and Telecommunication Authorities will receive a large slice of the definition-phase contract.

The ministers took a positive view of the proposed location of ESRF at Grenoble and have asked the European Commission to consider possible participation. This move could help to ease present problems over the siting of ESRF (see *Nature* 14 March, p.125 and 28 March, p.305), although since the facility has been proposed

in the context of the European Science Foundation, it does not formally come under the jurisdiction of the European Community. But because the problems of the siting of ESRF involve rivalry between member states (Denmark, Italy and France plus West Germany) and because the ministers of the Community long ago agreed to some measure of coordination between national policies and a definition of joint interests in science and technology, the research ministers agreed to set up a general information and consultation procedure between the ten member states and the Commission.

This proposal, from the Italian president, seems to have met with general approval, both from the larger countries such as the United Kingdom, whose stakes include the Neutron Spallation Source at the Rutherford Appleton Laboratory, and the small countries that fear being left out of the running for important projects and which want fairer geographical distribution

of scientific research sites. West Germany, however, raised the question of just how much access to major research institutes would have to be involved. As a first step, between now and the end of the year, the European Commission is to draw up an inventory of national and intergovernmental scientific laboratories and equipment.

Ministers also considered the Italian-backed Initiative for Research in Informatics applied to Society (IRIS). While the idea has the support of member states, they are not yet prepared to pay for it. Meanwhile, the Commission is to carry out a study of IRIS and to hold a seminar on it in December 1985.

Inevitably the French-backed proposal for concerted research in advanced technologies in Europe (Eureka) also came up for discussion at the research council. But apart from admitting that cooperation will include non-Community countries, officials intend to remain tight-lipped until the Milan summit, at the end of June, where European science and technology will be high on the agenda and at which it is hoped that a series of priorities will be agreed.

Anna Lubinska

US veterans

Leukaemia figures in doubt

Washington

A HIGHER than expected leukaemia mortality rate among military veterans who took part in an atomic test in 1957 seems to be a statistical anomaly, according to a National Academy of Sciences study* that examined mortality rates of veterans who were exposed to similar amounts of radiation in other tests. None of these other groups show a statistically significant increase in leukaemia mortality as compared with the general population.

An earlier study by the Centers for Disease Control had confirmed claims by veterans that an unusually large number of men who took part in the 1957 test code-named Smoky at the Nevada Test Site were dying of leukaemia. But the academy study suggests that chance alone could explain the result.

Approximately 200,000 servicemen participated in atmospheric nuclear tests from 1946 to 1962 at the Nevada site and in the Pacific. The academy study looked at 46,000 who took part in five series of tests in which radiation dosimeter badges were issued to the men.

A review of death certificates and medical records revealed that 10 of the 3,554 men present at the SMOKY test had died of leukaemia by 1982 — 2.5 times more than would have been expected for a similar group in the general population. But the study notes that when leukaemia deaths in the Smiky group are compared

with the leukaemia deaths in all five groups, the difference is not statistically significant ($P=0.16$). In fact, the total number of leukaemia deaths (46) in the non-Smoky groups was slightly less than expected (52). Likewise, the number of deaths from all cancers in the Smoky group (67) was less than expected (84). Data from the dosimeter badges implies that there should have been only 0.2 excess leukaemia deaths in the Smoky group. So if chance is not the explanation for the leukaemia deaths, then either the Smoky dosimeter data are several times too low or the generally accepted estimates of radiation-induced leukaemia dose-response curves are wrong.

Although the study notes that it is impossible to prove that chance alone explained the anomaly in the Smoky group, it points out that the more comparisons that are made, the more likely it is that one will seem to be statistically significant by chance. This is illustrated by the analysis of all five groups for eight different kinds of cancer. A small excess of prostate cancers is found in one group; but there is a one-third chance that when eight independent comparisons are made, at least one will produce a P -value of 0.5 just by chance.

Veterans' groups have called the report "ridiculous" and complain that it makes no sense to compare the atomic veterans with the general population. Because those selected for military service are healthier than the general population, such comparisons minimize health problems in the veterans.

Stephen Budiansky

*Mortality of Nuclear Weapons Test Participants, Medical Follow-Up Agency, National Research Council, 2101 Constitution Avenue, Washington DC 20418.

Energy

Conservation in vogue

FRENCH nuclear power is increasingly abundant, supplies of natural gas from Siberia and Algeria will soon outstrip demand and the French government has set up a study group to invent new ways of using electricity. So why does France need the Agence Française pour la Maitrise de l'Énergie (AFME), which last year spent some FF 2,500 million (£210 million) on supporting energy conservation and renewable energies in community housing, industry and research? Because it pays, a team of financial inspectors has concluded.

The inspectors were provided by finance minister, Pierre Bérégovoy, who is now tearing his hair out over the 1986 (election year) budget, due to go before parliament in November but to the Council of Ministers much sooner. Bérégovoy, who, like most finance ministers, is looking for savings, wondered if an increase in energy taxes could be arranged not only to increase revenue but also to save energy.

According to AFME sources who have had a chance to look over the inspectors' work, their conclusion — which is not due to be published — is that the "interventionism" represented by AFME is the best way of achieving energy savings. Moreover, AFME itself is the best way of going about the intervention.

AFME director-general M. Bernard Laponche says there has always been "tension" over AFME and its policy of investment aids. The tension arises between the "technical ministries" of industry, energy and research, backed by President Mitterrand himself, and the financial sources. Matters may have been made worse by last year's figures which show French primary energy consumption rising by 2 per cent despite AFME's investments, some 80 per cent of which go towards saving or substituting for oil.

But says the ex-trades unionist president of AFME, M. Michel Rolant, his agency's actions saved 2.2 million tons of oil equivalent (t.o.e.) in 1984 in a total of 191.6 million t.o.e., so things would have been worse without AFME. Moreover, the general export trade improved in France last year because of the cheap franc, so a little AFME juggling shows that the French energy bill went down last year — when measured in terms of months of general exports. In 1984, France worked just two and a half months to pay its energy import bill, the lowest since 1980.

In reality, it is still the cost of oil imports that makes AFME's work politically attractive. In its medium-term programme, AFME aimed to produce energy savings over the decade 1981-90 of 40 million t.o.e., more than a fifth of oil imports, of which some 20 million t.o.e. were to come from improving home insulation and 10 million from industry; renewables, which contributed 3.4 million t.o.e. in 1981, were

planned to bring in 10-14 million t.o.e. in 1990, mostly from biomass.

There is still a long way to go to reach those targets (set nearly three years ago) but AFME staff are encouraged by the fact that in 1984, for the first time, energy savings profited the balance of payments by more than AFME aid itself — FF 3,000 million against FF 2,500 million. Nevertheless, AFME aid is rarely 100 per cent, so that actual energy-saving or energy-substituting investments in France last year amounted to some FF 11,000 million. This sum may take a considerable time to pay off.

Robert Walgate

Soviet Union

Phobos mission

THE Soviet Union is planning the first-ever mission to Phobos, the larger and inner moon of Mars, in 1988. The two-probe mission will include a close fly-by of the satellite, at an altitude of 50m, during which a laser gun will be fired to stir up surface dust which will then be captured and analysed.

This will be the first Soviet mission to Mars since 1973. Eighteen months ago, *Aviation Week and Space Technology* suggested that the Soviet Union was planning a mission to Phobos, with an approach to within "a few thousand feet" for 1986.

The actual plans for the mission, at present code-named "Mission-F", seem considerably more sophisticated than the *Aviation Week* speculations, which suggested that the Soviets had abandoned Mars flights because of 14 failures or partial failures out of 15 launches or attempted launches between 1960 and 1973. The Soviets, they said, had realized that they could not compete with the plans for the US orbiter/lander mission. In the opinion of *Aviation Week*, the projected Phobos flight was simply a "poor man's asteroid mission".

Perhaps in answer to such smears, the Soviet space planners are trying to make the Phobos mission as international as possible. Sweden's Kiruna Geophysical Institute has been invited to supply an instrument for measuring the energy and mass of solar wind particles around Mars and Phobos. Poland will supply a device to study plasma-waves, similar to those supplied to the Venus-Halley probes now in flight. Bulgarian and West German scientists are developing a special laser mass spectrometer in conjunction with their Soviet colleagues; the East Germans will supply optical systems for filming and mapping and there are plans for a joint Soviet-French device for studying the chemical composition of the martian atmosphere.

Vera Rich

Chromosome libraries

Ploughshares from swords?

Los Alamos, New Mexico

AN ambitious project to create the first human chromosome-specific gene libraries is expected to have covered the entire genome by the end of the year. The libraries, which are being constructed at Los Alamos and Lawrence Livermore National Laboratories, are already attracting strong commercial and scientific interest. Once the project is complete, the rate of human gene mapping will increase by a factor of ten, according to the project team; batteries of probes for chromosome-specific diseases and chromosome-specific stains are among the other benefits expected.

Twelve commercial companies are among the 500 organizations that have already received chromosome libraries from the National Laboratory Gene Library Project. Investigators who receive libraries (free of charge) are asked to inform the project of whatever characterizations they make. The libraries so far available are made using two different restriction enzymes — *HindIII* at Livermore and *EcoRI* at Los Alamos. The cloned DNA fragments are up to 9 kilobases long. Now it is planned to produce 17-kilobase fragments, which are more likely to contain entire functioning genes and will be of greater use in basic molecular biology. Lawrence Livermore and Los Alamos National Laboratories are primarily weapons laboratories and not the most obvious places to find a cloning project. According to Larry Deaven, principal project investigator at Los Alamos, the laboratories were chosen because of their expertise in chromosome sorting, which arose from studies of the effects of radiation on animal cells.

The chromosomes for the libraries are extracted either from human fibroblast cell lines or from hybrid human/hamster cells. After treatment with two fluorescent dyes, different chromosomes are separated automatically under laser light. Chromosomes that are not easily distinguished with two dyes are subjected to a third stain: according to Deaven, the chromosomes that are easy to separate have now all been cloned and "we're left with the hard ones". The efficiency of cloning has improved dramatically since the project started in 1983: whereas several million chromosomes were then needed to start a library, the figure is now down to 100,000.

The library project, at present supported by the Department of Energy with a budget of \$400,000 this year, will not always remain at Los Alamos and Livermore. As soon as both phases have been successfully completed, stocks will be handed over to the National Institutes of Health, which will also maintain information on the libraries.

Tim Beardsley

UK embryo research

Embryo protection bill resurfaces

IN an unprecedented attempt to use the complex rules of House of Commons procedures, supporters of Mr Enoch Powell's Unborn Children (Protection) Bill failed by only the narrowest of margins to reintroduce it into the legislative timetable last week.

The bill, which would effectively ban all experiments on human embryos, was given a second reading in the House of Commons earlier this year by a large majority of 172 (see *Nature* 313, 618; 1985). But, partly because of lack of official government support, the bill was "talked out" at its third reading on 3 May (see *Nature* 18 April p.573). Normally, a private member's bill would, at this stage, have no chance of further progress, so the government seemed set to proceed sedately with its own, comprehensive legislation on reproductive technology, based on the recommendations of the Warnock committee (see *Nature* 312, 389; 1984).

In an extraordinary fight back, however, Mr Andrew Bowden MP agreed to reintroduce Mr Powell's bill by exploiting his position at the head of the queue on private members' motions day, 7 June. He included the motion that the House of Lords should finish its scrutiny of the bill, if passed by the Commons, by 5 July, although such a request cannot be enforced.

Opponents of Mr Powell's bill, including the Archbishop of York, re-argued their case in the days leading up to the protracted debate. Dame Mary Warnock saw a "great tide of moral fundamentalism, sweeping across from America" as a genuine threat to proponents of licensed, controlled research. In fact the question was settled in parliament by procedural means. Mr Dennis Skinner MP found and made use of a rule that a motion to make a writ for a by-election (there is one outstanding) takes precedence over any other business, and ensured that the subsequent debate on the subject took hours rather than the usual few minutes. Only 16 minutes of time remained to debate Mr Powell's bill and this was ruled as inadequate to put the case for a debate, which otherwise might have lasted all weekend.

Meanwhile, the Medical Research Council and the Royal College of Obstetricians and Gynaecologists last week pressed ahead with their voluntary licensing authority for *in vitro* fertilization (IVF) and embryology techniques (see *Nature* 4 April, p.397) by publishing the authority's guidelines. These are based on the Warnock committee recommendations but each research programme will be licensed and projects will be considered individually. Programmes involving genetic manipulation or implantation of human embryos in other species will be refused licences. The conditions for receiving a licence include:

- Scientifically sound research on the pro-

cesses and products of IVF.

- Sound reasons why human, rather than animal, tissue is to be used.

- The aim of research must be clearly defined and relevant to clinical problems.

- Pre-embryos (dividing cells up to determination of the primitive streak) resulting from the research should not be transferred to a uterus except to establish a particular pregnancy.

- Signed consent for the use of ova and sperm to be obtained from both donors.

- Fertilized human ova not to be cultured *in situ* for more than 14 days.

- The means of embryo disposal to be specified at the outset.

Nuclear winter

Cautious support from SCOPE

INVESTIGATIONS into the environmental consequences of nuclear war by the International Council of Scientific Union's Scientific Committee on Problems of the Environment (SCOPE), whose report will be published in September, essentially confirm the results, and reflect the uncertainties, highlighted by the US National Academy of Sciences (NAS) last year (*Nature* 312, 683; 1985). The likelihood of a nuclear winter — a prolonged period of sub-zero temperatures in mid-northern latitudes following a major exchange of nuclear weapons — is thus given further support. This was the message that emerged at the 16th and final workshop of SCOPE-ENUWAR, held at the University of Essex this week and last week. But SCOPE has taken investigations a step further by considering the possible ecological consequences of the potential climatic effects.

Paul Crutzen, of the Max-Planck Institut für Chemie, a member of the steering committee, emphasized that the SCOPE study differs from its predecessors in that, instead of defining an exact war scenario as a starting point, such as the 6,500 megaton baseline case adopted by NAS, it has considered the destruction of a variety of proportions of the available combustible material and the injection of tonnages of smoke into the atmosphere consistent with a several thousand megaton nuclear war. Taken together with the improved general climatic models which now incorporate, in three dimensions, the interactive feedback between smoke plumes and winds, this should enhance the credibility of suggestions of a nuclear winter, according to S. Schneider of the US National Center for Atmospheric Research.

What has become clear, said Crutzen, is that the proportion of soot produced in urban and forest fires following a massive nuclear attack — the "blackness" of the smoke — is perhaps the most important factor. This in turn is dependent on the type

The voluntary guidelines are particularly appropriate now, as the government has announced that it will not introduce legislation in the next parliamentary session — that is, for at least a year. The popular support for Mr Powell's bill means that the government would be under considerable pressure to include his proposal in any legislation it seeks to introduce.

The tortuous progress of Mr Powell's bill contrasts strikingly with the smooth passage of Mr Norman Fowler's emergency bill to ban commercial surrogacy, which received three readings in the House of Commons between 28 March and 13 May. It is due for a second reading the House of Lords on June 14. The bill bans commercial surrogacy agencies and advertising of and for surrogacy services.

Maxine Clarke

of fires foreseen, with urban and industrial areas being more significant than forest fires. At the end of the day, SCOPE's "baseline case" of an attack on 30 per cent of the combustible material in the developed world produces less smoke than the 6,500 megaton borderline case of NAS, but it is blacker, so the resulting effective optical depth is about the same (4.5 for SCOPE, 5.2 for NAS).

The most important unknowns in this estimate are the nature of the smoke from burning oil stocks, and the physical and chemical processes that could affect particles in individual smoke plumes. Little is known of what happens when an oil pool is set on fire, although experiments with pools up to 50m across suggest that the degree of ventilation is the crucial factor in determining the relative proportions of incompletely burnt hydrocarbons, elemental carbon and gaseous oxides, that is, how dirty the smoke will be.

The NAS and SCOPE studies both highlight the lack of knowledge of processes operating in discrete plumes (up to 50 km across). If mesoscale processes tend to cause particles to agglomerate, this will clearly increase the rate of removal by gravity ("rainout") and may affect the optical properties of smoke plumes. Discussions at the workshop focused on two possibly opposed effects. First, the interaction of the plume with local weather conditions may produce more precipitation than is usually foreseen, which could wash the particles out or cause agglomeration, thereby decreasing the amount of smoke reaching the stratosphere. On the other hand, meso and microscale processes within plumes may increase the lofting of smoke, thus putting more particles into the upper atmosphere.

As Barrie Pittcock of CSIRO Atmospheric Research, Australia, stressed, escape of soot into the stratosphere could cause marked climatic effects in the

Southern Hemisphere, especially if the war occurred in July rather than January. In particular, interactive climatic modelling suggests a lowering of tropopause (the boundary between the troposphere and the overlying stratosphere) from its present heights of roughly 9 km in high latitudes and 20 km over the tropics to about 4 km over the North Pole and 10 km in the Southern Hemisphere. This, in turn, would mean that a considerable amount of smoke, perhaps 60 million tonnes, enters the stratosphere where it has two climatic effects. First with an optical thickness of 0.1 to 0.5, the smoke results in concomitant reduction in global insolation of roughly 20 per cent. Second, a July war would cause some smoke to drift into the Southern Hemisphere, with the general consequence of reduced global precipitation. In particular, cooling over the Tibet plateau turns off the southwest monsoon. But it has to be emphasized that there remain significant uncertainties in such calculations because the models do not take account of the potential effects of precipitation.

Although much more research is needed into the implications of these climatic changes for both natural ecosystems and agriculture, the results give cause for concern. The areas likely to suffer the worst effects of nuclear winter, the tundra and boreal forests, are the best equipped to cope, noted T.C. Hutchinson of the University of Toronto, because they are naturally adapted to extremes of climate. But the tropical mangrove and evergreen rain forests have no such protection and are very vulnerable to damage by sub-zero temperatures lasting only a few days or weeks.

The effects on agriculture could be even worse, said M.A. Harwell (of Cornell University). What emerges is that although a July war would have more severe immediate and short-term climatic effects than one fought in January, the precise timing may be important. For example the occurrence of just one or two nights with temperatures below 15°C at a critical period in the growing season could wipe out an entire rice harvest. And perhaps as little as a 3°C fall in temperature would be sufficient almost to eliminate Canadian wheat production. This loss of production, combined with the likely absence of imports, would be disastrous for most of the surviving world population. For example, on loss of imports alone, even assuming 100 per cent of present production, Japan could only feed half of its present population. And most underdeveloped countries appear to have only about four months supply of stored food. If the climatic effects of a nuclear winter in July lie towards the colder end of the range physicists consider plausible, the ensuing lack of food may have a greater impact on the global population than the immediate effects of the nuclear attacks.

Peter Gambles

Sakharov

Mystery on the cards

RELATIVES of Academician Andrei Sakharov living in the United States are becoming increasingly concerned about his whereabouts and well-being. They know of nobody who has seen either Sakharov or his wife, Elena Bonner, in their Gor'kii exile, since February. During April, they received reports that Sakharov had begun another protest fast, and that he was conveyed to hospital for forced feeding on 21 April. A postcard signed by the couple (see illustration) also apparently dated 21 April, which represents them as commencing their spring-cleaning on that date, now proves to have been tampered with in transmission to conceal an original date of 1 April.

During the past two months, there have been many conflicting reports about the Sakharovs, including rumours that they were to be released to the West under an amnesty to mark the 40th anniversary of the end of the Second World War in Europe, and even that they had already arrived in Zurich. (These rumours may have emanated from Soviet official sources, in order to defuse international tensions before the Ottawa Human Rights "Helsinki Review" meeting.) Sakharov was also said to be threatening to resign

The 1 April or 21 April card.

from the Academy of Sciences in protest at the passivity of his fellow academicians regarding his plight and that of his wife, who has been refused permission to travel to the West for eye surgery. The arrival on 25 May of the "21 April" postcard initially led the relatives in Boston to have "serious doubts" about the protest fast, and to be more optimistic.

Closer examination, however, revealed a number of anomalies. Because mail from the Soviet Union frequently fails to arrive, Mrs Bonner numbers all postcards (the couple are apparently not allowed to write letters). The card dated "21 April" was numbered 21, but the family had previously received cards numbered 22 (dated 3 April) and 26 (dated 17 April). The latter took two

weeks to arrive, while card number 21 apparently took 34 days. Moreover, on card no. 21 Mrs Bonner asked if the family had received the birthday presents that she had sent [marked "4" in illustration] while card 22 (3 April) says: "the day before yesterday we received a card from mother saying that the birthday presents for Sasha and Tanya have arrived".

Furthermore, card 21 contains an odd sentence (with a grammatical error in the verb-form) which seems to imply that the snow had melted (past tense) [3] and the gutters were now running with water (present tense). In card 22 (3 April), Mrs Bonner had reported the snow almost all gone, and according to the *Pravda* weather reports there was no snow in the Gor'kii area during the last ten days of April.

The relatives therefore took the postcard, and the thirteen other cards received from the Sakharovs this year, to Charles Gershin, a certified master graphoanalyst in Massachusetts. Gershin knows no Russian, but the Russian cursive script, unlike printed Cyrillic, is based on the Latin alphabet. Examination under high-power magnification, therefore, made it possible for Gershin to detect interference. He found that on card 21:

- A "2" had been inserted before the date [1] which had originally read 1 April.
- The alteration of a single letter in line 2 had changed an original "April has gone" [2] (somewhat premature if written on the 21st) to "April has come".
- Similarly the alteration of one letter in the sentence about the snow changes an original "is melting" to "has melted" (with an unavoidable grammatical error).

The implication, therefore, is that the card number was tampered with in order to give the impression that the Sakharovs were leading their "normal" lives on 21 April, from which the logical conclusion would be that something untoward must have occurred. The unconfirmed reports of Sakharov's alleged fast state that he began it on 16 April, so how would the "tamperers" have had to hand the card mailed on 1 April? One possibility is that, for some reason, the censors had decided to withhold this card anyway, and then, when the story of forced-feeding broke on 15 May, the security authorities quickly looked through their stock of recent cards for anything that could be doctored.

The relatives in Boston are now unwilling to commit themselves on any report about Sakharovs, even whether they are alive or dead. According to Dr Efrem Yankelevich, Mrs Bonner's son-in-law, several friends in Moscow apparently received messages from the Sakharovs on 20 May (the eve of Dr Sakharov's birthday), but these were sent by facsimile, which, he says bitterly, "are even easier to forge than a postcard".

Vera Rich

Velikovsky defended

SIR — Yet another book and book review (*Nature* 25 April, p.692) on Velikovsky! Many people who have been warned off reading him by earlier writings must wonder how long this will go on and why. Are there really people who read Velikovsky, and if so, why would anybody with sense read so poorly recommended an author? Perhaps my experience will provide some indication.

I never dreamed of reading Velikovsky for ten years after his first book, *Worlds in Collision*, because the publisher's ill-considered advertisement suggested it was written in support of religious literalism, and scientific critics, rather rabid sounding but presumably competent, spoke unfavourably of him. I only became curious and started to read him when, as a commuter in need of reading matter, I saw some literary figure quoted to the effect that Velikovsky was much a better writer than his critics. He does indeed read well. His sentences flow, one paragraph leads into another and there is never a moment of boredom at which to break off. The catastrophes are vividly described, the reasons advanced for believing in them interesting.

The reasons may, of course, be quite wrong, but that uncertainty is part of the charm of such reading. If a Velikovskyan catastrophe seems an implausible explanation of the sudden death of the Siberian mammoths, one is at least impelled to wonder again what kind of catastrophe it was. If Velikovsky, in spite of some mildly favourable remarks by Einstein, seems unlikely to have discovered electrostatic forces neglected by the astronomers, one becomes curious to know the basic physics, the degree of unanimity of the astronomers, earlier theories (if any) of electrostatics in astronomy, whether, as Velikovsky claims, the recent space probes tell something relevant and whether Einstein had a habit of encouraging eccentrics.

Critics have made much of Velikovsky's alleged appeal to the ignorant and also to his supposed religious motivation, something never documented and which I do not find in his books. He continues to be read because he appeals to the intellect and the imagination, and to a hunger for good writing.

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Arms talks

SIR — Your leading article (*Nature* 21 March, p.205) about the Geneva arms talks is a sensible argument about strategies for the negotiations. You recommend that expectations not be built too high too soon, and I concur wholeheartedly. These are the most difficult negotiations we have entered into with the Soviets, because there are three subgroups on space and defence, intermediate-range nuclear weapons and

strategic weapons.

These negotiations will take years. Interim agreements along the way would be reasonable objectives. Both sides must be willing to pursue confidence-building measures designed to lessen tensions, and to give hope to people living under each flag that a period of lessening fear is possible, even probable. Improving the atmosphere for negotiations by ratifying the two unratified testing treaties might also be helpful. And trying to understand the issues from the other fellow's side of the table is sound advice indeed. To do that, we need to comprehend more about the other fellow, and even more than that, we need to know the fears, hopes and dreams of his countrymen.

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Cuts in wrong place

SIR — I have recently been following the debate over the Natural Environment Research Council (NERC) corporate plan in your pages. I have been interested in the work of some of the NERC institutes, particularly that undertaken by the Institute of Marine Environmental Research (IMER), the Freshwater Biology Association (FBA) and the Institute of Terrestrial Ecology (ITE), all of which are represented in the area in which I am presently but temporarily in residence.

I teach, and have taught, environmental science subjects in colleges and univer-

NERC staff reductions (from annual report)				
	1980-81	1981-82	1982-83	1983-84
HQ	191	191	191	186
NSS*	200	208	190	203
Institutes	2,870	2,750	2,678	2,376

Staff levels (1983-84) as percentage of 1980-81		
	% of 1980-81	% Change since 1980-81
HQ	97	-2.6
NSS	101	+1.5
Institutes	83	-17.2

*Excluding ships' crews.

sities in the United States, Canada and New Zealand where I took a similar interest in studies of equivalent organizations. I have found the work of the NERC institutes with which I am familiar to be generally of as high, if not higher, calibre as that in other countries. I was therefore surprised to learn of their present financial predicament. This led me to examine the NERC annual reports which contain the research council's record of diminishing manning levels between 1980 and 1984 (see table). The institutes (plus the British Antarctic Survey, BAS) have suffered a 17.2 per cent drop in manning levels against a 2.6 per cent drop in headquarters (HQ) staff and an actual increase in the NERC Scientific Services (NSS) of 1.5 per cent.

HQ and NSS contribute no commission-

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ed revenue towards the NERC budget whereas the institutes (not including BAS) do offset some of their costs through commissions. As it is also true that NSS is largely administrative in composition, this seems to be a rather good example of the tail wagging the dog. Surely cutbacks should be at least as heavy in the administrative sector (if not greater) as at the business end of the organization? That is certainly a difference between the way things are here in the United Kingdom and in the other countries I have mentioned.

It seems very sad, in light of this, that 1,000 scientific jobs are to disappear when, as seems probable, there should be some slack in administrative staff numbers yet to be taken up.

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Value-free science

SIR — Your correspondent M. Diesendorf (*Nature* 10 January, p.92 and 25 April, p.666) writes as if he believes complexity to be an adequate substitute for validity. If he does so believe he is in plentiful company, but errs nevertheless. Of course LD₅₀s are statistical statements; of course it is sometimes hard to isolate the cause of a particular death; of course juries are sometimes flummoxed. So what? Are "values" supposed to creep in under a sort of smokescreen?

As for the suggestion that the statement "I am going to poison my wife" may be "value-free" because it declares intent, let us recall the wise saying that there are times when one good laugh is worth ten thousand arguments.

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Mass extinctions

SIR — R.C. Hope (*Nature* 18 April, p.574) complains that he has had enough of the wild speculations of recent years about the extinction of the dinosaurs. As a palaeontologist, I heartily agree.

However, Mr Hope thinks that it is the palaeontologists who are to blame. If he examines the credentials of the speculators, he will find that the great majority of them are not palaeontologists. In offering his own speculation (that the mammals destroyed all of the vegetation and ate the eggs of the dinosaurs faster than they could lay them), he falls into the class of people whom he wishes to criticize. No palaeontologist would subscribe to such a simplistic and biologically unrealistic notion.

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Tuning in to neurotransmitters

The slow-acting chemicals that modulate fast synaptic transmission in the nervous system may help to unify the disparate disciplines that make up neurobiology.

For too long it has been common to make the analogy between the central nervous system (CNS) and digital computers. Both are hard wired, and information is transmitted as digitally coded electrical signals. But in the nervous system communication at the synapses between the neurones is a graded release of chemical neurotransmitters, which have fast, brief effects on very limited targets. In the past ten years it has been realized that an enormous number of substances are involved in synaptic communication, subtly modulating the transmission process.

Unlike the fast transmitters, most of the neuromodulators are slow acting, long lasting and often work at long range in very complex ways. A new concept of the nervous system is emerging of hard-wired circuits constantly tuned by chemical signals. This modulation gives the nervous system its flexibility and adaptability to long-term changes, both internal and environmental.

The distinction between the fast transmitters and the neuromodulators was explored on 16–17 May at the symposium that inaugurated the Merck, Sharpe and Dohme Neurosciences Research Centre at Terlings Park, Harlow, United Kingdom. Fast transmitters generally open ion channels in the postsynaptic membrane, thus changing the membrane potential. Onset is rapid and duration brief. As well as acetylcholine at the neuromuscular junction, in the CNS the amino acids glutamate and aspartate seem also to be fast excitatory transmitters, whereas gamma-aminobutyric acid and glycine are inhibitory. Modulators, which include the catecholamines noradrenaline, dopamine and serotonin and numerous neuropeptides, act metabolically, like hormones, by activating a chemical second messenger in the postsynaptic cell.

But even this classification is breaking down: it is the postsynaptic receptor that determines the action of a transmitter, not the chemical. Most transmitters have more than one type of receptor; for example acetylcholine has a fast receptor associated with an ion channel and a slow receptor that works through a chemical second messenger. Both can be present on the same cell, so one transmitter can elicit both fast (milliseconds) and slow (seconds or minutes) changes in the membrane potential.

Nor do modulator chemicals all work through the same second messenger. Some alter the activity of adenylyl cyclase which

in turn changes the level of cyclic AMP; others stimulate the breakdown of phosphatidyl inositides (for review see Berridge, M.J. & Irvine, R.F. *Nature* **312**, 315; 1984). Again the pathway is determined by the receptors and several modulators have two types of receptor, one linked to each pathway.

Receptors for neuromodulators may be on any part of the neurone. Modulators interacting with receptors on presynaptic terminals, seem to modulate transmitter release, possibly by altering calcium flux. Other specific actions of neuromodulators include the suppression or enhancement of various postsynaptic ionic currents, which alter the excitability of the postsynaptic membrane (see *Nature News and Views*, 6 June, p. 454). Some of these effects seem to be mediated by cyclic AMP-dependent activation of protein kinases (S. Waalas, Rockefeller; Nestler, E. & Greengard, P. *Nature* **305**, 583; 1983). Protein phosphorylation could also be involved in the control of synaptic vesicle release; in short-term memory by altering synaptic efficacy; and in regulation of gene expression. The resulting changes in protein synthesis underlie long-term memory, by producing permanent changes at synapses, and in regulating receptor density, which might alter a neurone's sensitivity to transmitters.

Many modulators do not seem to be released at typical synapses but as a cloud that diffuses through the extracellular space to targets that may be over 100 μm away. Here again it seems to be the specific location of receptors that determines which cells respond to such 'parasympaptic' diffuse signals (F.O. Schmitt, Massachusetts Institute of Technology), creating order in place of potential chaos. Unless there is a predictable, structurally defined basis to the CNS, precise responses to such imprecise signals cannot be expected.

Modulators interact not only with fast transmitters but also with each other. Some neuropeptides, such as vasoactive intestinal polypeptide (VIP), work partly by increasing neuronal metabolism and possibly also by increasing local blood flow. Based on evidence for amplification of the action of noradrenaline by VIP, F. Bloom (Scripps, La Jolla) proposed that such interaction could produce very precise spatial control of cortical circuits. The mosaic of VIP-containing cells in the cortex may generate a localized, activity-dependent modulation of the global effects of noradrenaline, so regulating activity in single minicolumns of

neurones only 30 μm in diameter.

Although we are beginning to glimpse the complexity of neuromodulator effects at the cellular level, we know even less about the effect of neuromodulators on behaviour. The most dramatic analysis comes from E. Kravitz and his colleagues (Harvard; Kravitz, E. *et al.* in *Model Neural Networks and Behavior*, ed. Selverston, A. Plenum, New York; 1985) on the control of the reciprocal aggressive and submissive postures in the lobster. In the aggressive posture, all the flexors muscles are contracted; in the submissive, the extensors contract. Both postures can be elicited by injections of monoamine. Serotonin produces the aggressive posture, octopamine the submissive. These modulators act both in the periphery and in the CNS. At the neuromuscular junction both have similar actions producing long-lasting increases in transmitter release and enhancing muscle contractility. In the CNS, however, they have opposing effects on specific groups of motor neurones. The neuronal programmes that result in the two postures are hard wired but the readout of the appropriate programme seems to be facilitated by the particular modulator as it raises or lowers the thresholds of the relevant motor neurones (at least). The cellular mechanisms underlying the serotonin effects in the periphery are already being analysed and the central effects could be pursued to the cellular level. What causes the initial changes in modulator concentrations remains an open question.

It is a serious problem for neurobiological research that the subject is studied on a number of different levels, ranging from the molecular constituents of membranes to whole animal behaviour, and it is difficult to access one level from another. The lobster studies are a good example of the power of neuropharmacology to provide a bridge between these levels. In order to understand how neuronal circuits work, it is now necessary to appreciate the way a chemical messenger can cause short or long-term changes in receptors or channels, which in turn can influence a neurone's excitability, change its metabolism, modify transmitter release or affect the synthesis of new proteins, including receptors.

We are only just beginning to understand these processes, but it seems that the ever-increasing list of neuroactive substances and the complexity of their actions may be the unifying factor so far lacking in neurobiology.

Jennifer Altman

Taxonomy

How many unknown species are yet to be discovered?

from Jared M. Diamond

IN THE nineteenth century, when much of the globe was still biologically unexplored, the discovery of new species was the most exciting and productive area of biology. Today, the study of molecules is the trend, and searches for new species are outmoded. The most vocal searchers, the cryptozoologists, are ridiculed because of their fixation on finding mythical large species — preferably primates, carnivores or long-extinct taxa such as dinosaurs¹.

No one doubts, and few people care, that thousands of small terrestrial invertebrates, cold-blooded vertebrates and plants await description, especially in the tropics. These groups have even yielded major surprises, such as the discovery of the world's largest lizard (the three-metre-long Komodo dragon) in Indonesia in 1912, or of the dawn redwood tree (*Metasequoia*) in China in the 1940s. Nor does anyone question that novelties occur in the abysses of oceans: for example, recent finds of a new family of large-mouthed shark, living crossopterygian fish and a primitive class of molluscs (monoplacophorans) previously thought to have become extinct about 400 million years ago.

Some biologists, undeterred by being unfashionable, continue to scour the Earth for new birds and mammals — but are there any more species to be found? The answer is yes: new birds and mammals turn up every year and occasionally even a species thought extinct is rediscovered. Although not satisfying the cryptozoologists' desire for monsters, these finds have proved important in several branches of biology^{1,2}. Tables 1 and 2, assembled from the recent taxonomic literature³⁻¹², summarize the new finds by region, date and taxon.

Since 1900, 134 accepted new genera of living mammals have been discovered³⁻⁶, mostly in the tropics (Table 1), to yield a current total of around 1,050. From its nineteenth century peak the discovery rate dropped steeply to an asymptote of about 1 genus per year, maintained since 1940 (Table 1). No new genus has been found in the United States since 1916 (the last being the doubtfully distinct bat *Idionycteris*), and none in Europe since 1922 (the vole *Dinaromys* in Yugoslavia).

The 20 mammalian orders are represented unequally in the new finds (Table 2). Eleven orders, all of them poor in species, have contributed no new genera, whereas four (rodents, bats, shrews and marsupials) have produced 90 per cent of the novelties. Re-expressing the results as the ratio of new genera to total number of species to take account of differences in

richness of species among orders, these last four orders are still the most abundant, joined by rabbits and whales (new genera/total number of species is > 2.8 per cent). Among the species-rich orders, primates and carnivores have yielded no new genera since 1907 and 1919, respectively. Thus, large terrestrial mammals seem to be almost completely known at the genus level; the recent new species are all to be found among small terrestrial mammals and whales. In fact, no new species of a large terrestrial mammal has turned up since the kouprey, a primitive, rinderpest-resistant species of wild ox, discovered in Indochina in 1937 (ref. 13).

Small does not mean unimportant. Recent finds include two mammals so distinct that they had to be placed in new families. One of these, the bumblebee bat (*Craseonycteris*), found in Thailand in 1974, is the smallest known warm-blooded animal (see figure)¹³. With a weight of only 2 grams and a length of 3 cm, it challenges physiologists to understand how it reconciles small size with heat conservation. The other new family is the dormouse *Selevinia*, found in Kazakhstan deserts, which has the peculiarity of moulting its epidermis as well as its hair⁵.

Several genera of supposedly extinct late Pleistocene mammals have surprised zoologists by turning up alive. The Australian mountain pygmy possum (*Burramys*), described in 1896 from fossils 20,000 years

old, was caught rummaging behind the garbage pail in the kitchen of Melbourne University's ski lodge in 1966. The 'ice-age peccary' *Catagonus* is now the largest of living peccaries, having been found running in herds through the Paraguayan Chaco¹⁴. Bulmer's fruit bat *Aproteles* was described from 10,000-year-old fossils, then discovered alive in a New Guinea cave, and finally re-exterminated or driven out by hunters, all within the same year¹⁵.

Among birds only nine new genera (but 134 new species) have been found since 1934 (refs 7-12). As with mammals, the discovery rate plummeted in the first decades of the twentieth century, but has remained steady at around three species per year since 1941 (Table 1). Most of the novelties have come from the neotropics and Africa, with bursts of discoveries occurring when the mountains of New Guinea and the Philippines were explored between 1938 and 1962. In recent years South America, particularly Peru, has been responsible for about half the novelties. Neither Europe nor the United States has yielded a new species in a long time. Disproportionately few of the new species belong to big, conspicuous and wide-ranging orders of birds such as hawks (new species since 1934/total species = 1/287), parrots (1/346), ducks (0/148), charadriiforms (waders and gulls, 0/334) and ciconiiforms (herons and ibis, 0/122). Disproportionately many belong to groups that tend to be cryptic, geographically localized or small, such as owls (4/146), petrels (3/66), tinamous (2/47) and babblers (10/255). Eight of the nine newly described genera are songbirds (passerines) with small geographical ranges.

Although most of the new bird species are similar to previously known species, several are very unexpected in distribution,

Table 1 New genera of living mammals described from 1900 to 1983 and new species of living birds described from 1934 to 1975

Region	Mammals	Birds
Neotropics	31	56
Africa	27	35
Oriental	20	8
Philippines	7	8
Celebes	6	0
New Guinea	19	9
Australia	4	3
Pacific islands	1	10
Palearctic	14	2
North America	2	0
Oceanic	3	3
Date		
1900-09	42	
1910-19	23	
1920-29	24	
1930-39	12	
1934-39		27
1940-49	7	27
1950-59	8	32
1960-69	9	31
1970-79	8	
1970-75		17
1980-82	1	

Table 2 Newly described genera of living mammals in each order

Order	New genera/total number of species
Monotremata	0/3
Marsupialia	11/260
Xenarthra	0/29
Insectivora	11/391
Scandentia	0/16
Dermoptera	0/2
Chiroptera	26/917
Primates	2/180
Carnivora	2/269
Cetacea	4/78
Sirenia	0/5
Proboscidea	0/2
Perissodactyla	0/18
Hyracoidea	0/7
Tubulidentata	0/1
Artiodactyla	3/185
Pholidota	0/7
Rodentia	72/1,752
Lagomorpha	3/65
Macroscelidea	0/19

The number of new genera are those described between 1900 and 1983; the total number of species are those found up to 1983.



The bumblebee bat *Craseonycteris thonglongyai*, the world's smallest mammal, was discovered in 1974 (photograph courtesy of J. McNeely).

morphology or behaviour. Peacocks were thought to be confined to southeast Asia until the discovery of the African peacock (*Afropavo*) in 1936. The tiny Philippine babbler *Micromacronus*, although in size no rival of the bumblebee bat, is still one of the smallest songbirds. The New Guinea bowerbird *Archboldia* proved to have a uniquely sadomasochistic display: the male crawls prostrate on the bower floor while the female whips her wings over him¹⁶.

In contrast to the case for mammals, there seems to be no bird species described as a fossil and then found alive. But several species thought to be extinct have been rediscovered after a few decades or, in one case (the Bermuda petrel), after three centuries. The most important such rediscovery was of New Zealand's kakapo, the largest and only flightless parrot in the world, and the only flightless bird with a lek mating system¹⁷. Other notable discoveries were those of the takahe, a flightless grazing gallinule in New Zealand's alpine grassland, and New Guinea's yellow-fronted bowerbird, whose ornate plumage

and spartan bower lend support to the transfer theory of bower evolution¹⁸.

In summary, finds of new birds and mammals will please neither cryptozoologists nor those who consider biological exploration an anachronistic waste of time. New species are continuing to turn up at a slow but steady rate, and many areas of biology are being enriched by the discoveries. But, ominously, the rate of extinction of birds and mammals is rising steeply, and will soon overtake the rate of discovery as the destruction of continental tropical rain forests proceeds. Few of the endangered tropical species will have been subjected to much field study before they vanish. Of the very few species thought extinct and subsequently rediscovered, almost all are now endangered. Man seems to find it much easier to bury species alive than to resurrect them permanently from the dead. □

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Mathematics

The power of positive thinking

from Ian Stewart

IN 1900 David Hilbert delivered a famous address to the International Congress of Mathematicians in Paris in which he discussed 23 open problems whose solution he considered to be of outstanding importance. C.N. Delzell has now improved the known results of the 17th of Hilbert's problems: the representation of positive functions as sums of squares. This lies at the heart of the algebraic theory of inequalities. For example, the inequality $x^2 + y^2 + z^2 \geq xy + yz + zx$ follows easily from the observation that $2(x^2 + y^2 + z^2 - xy - yz - zx) = (x-y)^2 + (y-z)^2 + (z-x)^2$. Hilbert's 17th problem effectively asks whether a similar trick is capable of proving any inequality involving only rational functions. A rational function is the quotient $f(x,y,z,...)/g(x,y,z,...)$ of two polynomials. If a rational function is positive for all real values of its variables,

must it be the sum of a finite number of squares of rational functions? Hilbert could prove it for functions of one or two variables, but not in general.

The first solution of Hilbert's 17th problem in the affirmative was obtained by E. Artin (*Abh. Math. Sem., Hamburg* **5**, 100; 1927). In the 1950s the solution was improved by A. Robinson, H. Henkin and G. Kreisel, who used ideas from model theory (the study of structures satisfying axiomatic conditions) and mathematical logic. Recently, Delzell (*Inventiones Mathematicae* **76**, 365; 1984) has refined the solution further, showing that it can be made continuous and constructive.

To understand Delzell's work, consider the simpler case of the Four Squares Theorem. J.-L. Lagrange proved in 1770 that every positive rational number is a sum of four rational squares. For instance,

$5/3 = 1^2 + (2/3)^2 + (1/3)^2 + (1/3)^2$. The proof was constructive, in the sense that it provided a definite process for finding the four squares required; but there was no obvious pattern to the final results. Small changes in the rational number concerned could lead to large changes in the summands. H. Heilbronn, however, proved the startling result that a uniform analytical solution exists (*J. London Math. Soc.* **39**, 72; 1964), in the following sense. There are four specific complex-analytical functions p, q, r and s , such that for all complex z the identity

$$z = p^2(z) + q^2(z) + r^2(z) + s^2(z)$$

holds. Further, whenever z is rational and positive, then each of $p(z)$, $q(z)$, $r(z)$ and $s(z)$ is rational. Thus, the same analytical formula provides a representation as a sum of squares for any positive rational z .

It is natural to enquire whether Hilbert's 17th problem has a similar type of solution. Because of the generality in which the problem is currently posed, the notion of an analytical solution must be replaced by that of a continuous solution: basically a fixed sum of squares of continuous functions. In 1982, Delzell produced some negative evidence showing that any representation of fourth-degree polynomials in three variables, as a sum of squares of second-degree polynomials, must be discontinuous.

This negative evidence, however, has a loophole. Nothing in the problem specifies that positive polynomials must be represented as sums of squares of polynomials. Indeed, if the problem were to be stated under this restriction, it would be false. T.S. Motzkin (in *Inequalities* ed. Shisha, O., 205, Academic, New York; 1967) has proved that $1 + x^2y^4 + x^4y^2 - 3x^2y^2$ is always positive, but cannot be written as a sum of squares of polynomials. (But it is a sum of squares of rational functions, thanks to some fortuitous cancellations of terms). And Delzell's current work exploits this loophole to obtain a constructive, continuous solution.

To state Delzell's theorem precisely would involve too much jargon, but its spirit is this: there exist specific continuous functions $p_1, p_2, \dots$ and $r_1, r_2, \dots$ such that every positive polynomial f can be written as a finite sum of terms $p_i(c, d, \dots, x, y, \dots) r_i^2(c, d, \dots, x, y, \dots)$. Here, p_i are positive numbers, r_i are rational functions, the terms $c, d, \dots$ are the coefficients of f and $x, y, \dots$ are its variables. The restriction to polynomial f is made to obtain a simple statement of the theorem. The result holds if the coefficients of f are real numbers or, more generally, lie in a 'real closed field'. The case of a general ordered field of coefficients remains open.

A good mathematical problem doesn't just die, nor does it merely fade away into oblivion, but remains a source of inspiration. Hilbert's 17th problem continues to be a part of living mathematics. □

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Astronomical clocks

Probing the early Universe with the millisecond pulsar

from Bernard Carr

COULD the millisecond pulsar known as PSR1937+21 provide information about processes that occurred within the first millisecond of the Big Bang? That is one of the intriguing questions raised by the results reported by M.M. Davis, J.H. Taylor, J.M. Weisberg and D.C. Backer on page 547 of this issue.

The millisecond pulsar was discovered in 1982 and immediately gained prominence as the pulsar with the shortest known period; according to theory, its signal must be produced by a neutron star rotating a thousand times a second. What promises to make PSR1937+21 such a useful cosmological probe, however, is that its rotation rate remains remarkably constant: apart from a steady and well-understood increase due to its energy emission, the rate has remained stable to one part in 10^{13} over two years. The pulsar thus provides an extraordinarily accurate clock, of relevance to cosmology in that many processes in the early Universe are expected to produce a background of gravitational waves, which should induce a tiny jitter in the clock's regularity.

To demonstrate the stability of the millisecond pulsar required considerable effort. The radio signal of the pulsar was timed initially with a rubidium clock at Arecibo Observatory but a multitude of corrections had to be applied before its regularity could be inferred. The rubidium measurements had first to be checked for any drifts relative to the master clock in Washington and then corrections had to be made for such effects as the relativistic delay induced by the gravitational field of the Sun, the changing Doppler shift associated with the Earth's motion through the Solar System and the dispersion produced by interplanetary and interstellar plasma. Most of these effects can be estimated with high accuracy and, after they were allowed for and a best fit made for the location, period and spin-down rate of the pulsar, the residual noise is only about one μ s over the observation period of two years. This is comparable with the accuracy of the best man-made atomic clocks. The residual noise may in part derive from the fact that the corrections mentioned above are imperfect. If so, improved estimates of these corrections could eventually reduce the residual noise to only 0.1 μ s. For the present discussion, the most interesting source of noise would be gravitational waves. If the waves have a dimensionless amplitude h and a period P (assumed to be less than the light-travel time to the pulsar), then they will change the arrival time of the pulses by an amount hP , an effect first

pointed out by S. Detweiler (*Astrophys. J.* **234**, 1100; 1979). For a continuous background of waves, this will induce a residual of no more than $h\tau$ over a time τ , because the pulsar is most sensitive to waves with $P > \tau$. (The effects of waves with $P > \tau$ would be largely absorbed into the fit to P and its derivative.) If the pulsar clock has an accuracy of Δt , it can be used to look for waves with period τ and amplitude $h = \Delta t/\tau$.

For PSR1937+21, the limiting amplitude is presently down to 10^{-14} , which means that one may already exclude a density of gravitational waves comparable with that in the microwave background ($\Omega_r \approx 10^{-4}$ when expressed in units of critical density). However, the limiting density scales as $(\Delta t)^2\tau^{-4}$ and so the sensitivity of the millisecond pulsar as a gravitational wave detector is likely to improve dramatically with time. For example, if the timing residual can be reduced to 0.1 μ s, and if this stability can be maintained over 10 yr, the pulsar could eventually search for a background of waves with density as low as $10^{-4}\Omega_r$.

The sensitivity of the millisecond pulsar is optimized for waves whose period is comparable with the observation time (a few years). Unfortunately, the most prolific sources of gravitational waves at the present epoch (supernovae and black holes) are likely to produce waves with periods much shorter than this. The one exception might be a population of binary black holes; these would produce radiation through quadrupole losses and could, in principle, be detectable if they were sufficiently numerous (J.R. Bond and B.J. Carr, *Mon. Not. R. astr. Soc.* **207**, 585; 1984). However, the most likely source of gravitational radiation in the required waveband would be processes that occurred in the early Universe.

This can be understood as follows. Cosmic processes which occurred at a time t after the Big Bang tend to produce radiation with a wavelength of the order of the horizon size at that time (ct); when one allows for redshift effects, this corresponds to a present period of about $(t_0 t)^{1/2} \approx (t/10^{-4}\text{ s})^{1/2}$ yr, where $t_0 \approx 10^{10}$ yr is the age of the present-day Universe. Thus, the millisecond pulsar can be used to look for waves generated at around 10^{-4} s, providing their amplitude is large enough. It turns out that the condition on the amplitude can be satisfied only if the waves are generated later than a time Δt after the Big Bang. The timing residual of PSR1937+21 thus directly determines how far back we can probe the history of the Universe.

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The period around 10^{-4} s is rather significant as it corresponds to the time of the cosmological phase transition at which free quarks transform into nucleons. Recently, E. Witten (*Phys. Rev. D* **30**, 272; 1984) has suggested a rather interesting way in which this phase transition could have generated gravitational waves. If the transition is first order and proceeds by the formation of bubbles which expand explosively, one would expect a gravitational wave background to be generated when the bubbles collide. It is not clear whether the amplitude of the waves would be large enough to be detected by the millisecond pulsar, but it is conceivable. Another consequence of this scenario is that the fraction of the Universe outside the bubbles would be compressed into invisible quark 'nuggets', which might be candidates for the dark matter in galactic halos.

Various other effects in the early Universe could produce a gravitational wave background. For example, if the Universe undergoes an inflationary phase (a period of exponential expansion) at some epoch, characterized by a Hubble rate H , then one expects quantum effects to generate waves with density $(H/M_p)^2\Omega_r$ over a wide period range; here M_p is the Planck mass. This effect was first pointed out by A.A. Starobinsky (*JETP Lett.* **30**, 682; 1979). Such an inflationary stage may occur at around 10^{-35} s after the Big Bang, according to the Grand Unified theories, as a result of the Universe getting trapped in a false vacuum state; it may also occur because of quantum gravity effects. In this situation, the waves detectable by the millisecond pulsar would not be generated at 10^{-4} s, but they would fall within the horizon at 10^{-4} s.

Another possibility is that the Universe could undergo spontaneous symmetry breaking at some epoch, resulting in the formation of cosmic strings. These strings can be thought of as topological defects which are trapped in the symmetrical state and, if some of them form closed loops, they should generate gravitational waves through oscillations. The spectrum of the waves expected is rather complicated (C.J. Hogan and M.J. Rees, *Nature* **311**, 109; 1984) but in the period range relevant to the millisecond pulsar it would be flat with a density of the order of $(T/M_p)\Omega_r$, where T is the temperature of the symmetry breaking. It has been proposed that sufficiently large loops could serve as seeds for galaxy formation (A. Vilenkin, *Phys. Rev. D* **24**, 2082; 1982); this suggestion is already close to being tested by using the millisecond pulsar to look for the associated gravity waves. Although there are other ways of detecting gravitational waves, such as laser interferometry, none is sensitive to waves with a period of a year. Therefore, given time, PSR1937+21 could serve as a unique cosmological probe. □

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Experimental petrology

Putting the squeeze on rocks

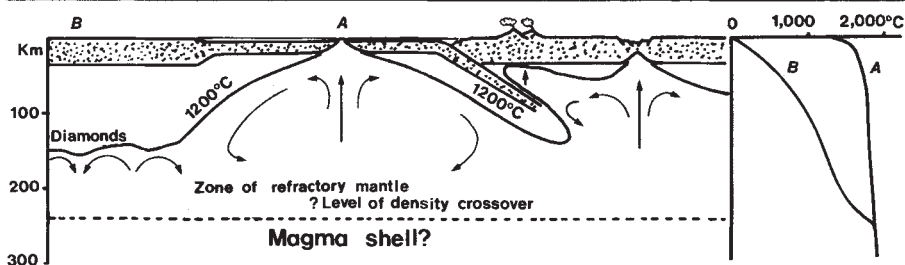
from E.G. Nisbet

CONTINENTS, oceans and the very air we breathe are the cumulative product of aeons of activity deep in the Earth's mantle. Although there are indirect means of investigation available, it would obviously be advantageous to be able to squash rocks in the laboratory to the high pressures and temperatures to which they are subjected in their natural environment. Unfortunately there are technical problems associated with this high-pressure route to the mantle. This has meant that questions such as the origin of komatiites and the controversial suggestions of an Archaean 'magma ocean' have remained unanswered. But recent improvements, especially in Japan¹, have enabled experimenters to attain pressures of up to 30 GPa (as a rule of thumb 1 GPa is equivalent to 30 km of rock) and simultaneously maintain temperatures well over 2,000°C. On page 566 of this issue, E. Takahashi and C.M. Scarfe² report results at pressures up to 14 GPa that have interesting consequences for models of the Archaean mantle and thus for the evolution of the Earth.

One of the most interesting problems is how komatiites originated. These lavas, commonly of Archaean age, are highly magnesian (up to 30 per cent MgO) and thus had high temperatures of eruption (over 1,650°C). Crude closed-system mass-balance calculations would suggest that they represent high degrees of partial melting of mantle; a more likely alternative is that they are the product of early melting in an open system at great depth. This suggests that, since the mantle must have been very hot, it was also fairly refractory. Until recently, most experimental work has been with apparatus capable of reaching pressures of 4–5 GPa (40–50 kbar) and 1,700–1,800°C. Both D.H. Green³ and M.J. Bickle⁴, for instance, have shown that natural komatiite magmas must indeed have been very hot. In addition, there have been various suggestions that at great depth the crystalline phases, such as olivine, are lighter than the melt^{5–7}. If such a density crossover existed, it may be that the Archaean mantle contained an 'ocean' of dense komatiitic magma at a pressure of about 8 GPa overlain by a thermal boundary layer of floating olivine crystals⁵.

These exotic models have provoked a flurry of experimental work using various techniques. At Lamont-Doherty Geological Observatory, E.M. Agee and D. Walker are carrying out experiments at 5–6 GPa and high temperatures, while at California Institute of Technology, shock measurements give strong circumstantial support to the proposed density crossover⁸. Using the 5,000 ton press at Okayama University, Takahashi and Scarfe² have melted peri-

dotite under pressures of up to 14 GPa. Their results show clearly that, as predicted⁹, early melts close to the peridotite solidus at 5–7 GPa and roughly 1,800°C are komatiitic with over 30 per cent MgO by weight. Equally nice is the confirmation that the slopes of the solidus and liquidus^{5–7,10} are roughly as expected, though there is considerable controversy about the extent to which they converge.



Cartoon illustrating aspects of some of the more richly speculative ideas about Archaean tectonics (see refs 5–7, 13, 14). Geotherms for locations A and B.

At high pressure, solidus and liquidus are probably sub-parallel and not greatly different from adiabat of the liquid¹⁰.

The consequences of all this for our models of the Archaean asthenosphere and lithosphere are considerable. Magma ocean models are strongly supported by circumstantial evidence but are as yet quite unproven; however the results reported by Takahashi and Scarfe place severe constraints on thermal and structural models of the asthenosphere. Not the least of these is the interesting contrast between the high temperatures implied by the experiments with komatiite and the strong evidence from diamonds for much cooler temperatures at depths of approximately 150 km beneath ancient continental nuclei. Diamonds have been produced in some numbers from Witwatersrand gold mines (McIver, personal communication) and are generally found in 'pipes' cutting through ancient continental crust. Inclusions in South African diamonds have been dated at roughly 3,300 Myr¹¹ (although the intrusive pipes are much younger). This would imply a thick continental lithosphere, at least under part of southern Africa, as far back as the Archaean. Taken together with the stability field for diamond, this in turn requires that part of the mid-Archaean subcontinental mantle must have been at a temperature of 900–1,300°C at 4–5.5 GPa¹². How can this be reconciled with the indications from Takahashi and Scarfe² of 1800°C at 5–7 GPa?

The most likely answer is that, rather than being uniform, the thermal structure of the mantle was strongly regionalized¹³, with much hotter conditions under oceanic areas than under continents. It may be that under the early continents the lithosphere

slowly thickened as the continent aged and cooled, eventually allowing diamonds to form and become frozen into the lithosphere. In contrast, the lithosphere under the oceans would have been much thinner. Regionalized models of this sort with relatively low sub-continental temperatures (1,200°C at 130 km depth) have previously been proposed⁵ to account for variations in the nature of erupted lavas. Perhaps under old (3,800–4,000 Myr) continents, the thermal base of the lithosphere was at about 150 km by the mid-Archaean (~3,000 Myr ago), while under zones of rifting and under oceanic areas the base was much shallower. Komatiitic liquids may have ascended in various tectonic settings,

ranging from rifting basins within continents to mid-oceanic ridges. Their generation may have ranged from low degrees of open-system partial melting of refractory asthenosphere at pressures of about 5 GPa, to tapping a putative magma ocean. Away from recently active zones of hot asthenosphere, the sub-continental lithosphere must have slowly cooled and thickened.

What next? The Japanese experiments are on the verge of tackling some of the most interesting problems in petrology. No doubt we shall soon know much more about the high-pressure shape of the solidus and liquidus curves and the density relationships at depth. Perhaps soon we will be rid of the uncomfortable notion⁵ that a magma shell existed in the Archaean asthenosphere: if it is disposed of we will be left with a better understanding of the mantle's history. If it survives, komatiites may have much to teach us about the chemical differentiation of the planet and the history of the lithosphere and exosphere. □

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Oncogenic intelligence

Oncogene activation by fusion of chromosomes in leukaemia

from Jerry M. Adams

AMONG the many chromosomal abnormalities associated with human neoplasms, the most frequent is the unusually small Philadelphia chromosome (Ph^1), named after the city in which it was discovered^{1,2}. The Ph^1 chromosome is present within all leukaemic cells in at least 90 per cent of patients with chronic myeloid leukaemia (CML), an invariably fatal disease characterized by insidious proliferation of the white blood cells², and results from a translocation (exchange of arms) between chromosomes 9 and 22. It has long been suspected that this event activates a cellular oncogene which then stimulates uncontrolled proliferation of the myeloid stem cell and its progeny. Increasingly over the past three years³⁻⁹, it is the cellular proto-oncogene *abl* (*c-abl*), identified by its relationship to the oncogene (*v-abl*) of the Abelson leukaemia virus of mice, that has been implicated in CML, but how the *abl* gene might be altered by the translocation has remained obscure. Now, reports by Shtivelman *et al.* on page 550 of this issue¹⁰ and by Heisterkamp *et al.* soon to be published in *Nature*¹¹, establish that the translocation fuses *c-abl* to another gene in such a way that a novel hybrid polypeptide can be made.

As illustrated on the left side of the figure, the Ph^1 chromosome is generated by a reciprocal exchange between chromosome 9, which bears the *c-abl* gene, and chromosome 22. The exchange always occurs at the same bands (q34 on chromosome 9 and q11 on chromosome 22) and yields an extended chromosome 9, 9q⁺, and Ph^1 , the shortened chromosome 22.

The *c-abl* gene was first associated with CML when the 9;22 translocation was shown to shift *abl* to the Ph^1 chromosome³. More importantly, the fusion region between chromosomes 9 and 22 was later cloned from one CML cell line and shown to involve the linkage of a region of chromosome 22 to sequences near the 5' end of *abl*. The region on chromosome 22 was subsequently denoted *bcr* (for breakpoint cluster region) because all chromosome 22 breakpoints associated with the 9;22 translocation fell within a 5.8-kilobase-pair (kb) segment⁵. Although breakpoints near the *c-abl* gene were rarely detected (for reasons that will become clear below), nevertheless a novel *abl* messenger RNA of about 8 kb was formed in CML cells as well as, or instead of, the normal 6-kb and 7-kb *abl* messengers. It seemed likely that the new mRNA was derived from the Ph^1 chromosome^{6,7} and an *abl* polypeptide with an altered amino(N)-terminal segment, presumably

translated from the new *abl* messenger, was also identified^{8,9}.

These results raised the intriguing possibility that the translocation fuses *c-abl* to a *bcr* gene, as now clearly established by Shtivelman *et al.*¹⁰ and Heisterkamp *et al.*¹¹. The emerging picture of the 9;22 translocation is outlined in the figure. The newly identified *bcr* gene¹¹ is oriented on chromosome 22 in the same direction as *c-abl* on chromosome 9. The breakpoints on chromosome 22 fall within a 5.8-kb region near the middle of the *bcr* gene, spanning two small exons (coding segments)¹¹. On the other hand, the chromosome 9 breakpoints may be scattered over a much wider region, as yet poorly defined but often more than 50 kb 5' to the *c-abl* gene^{4,11}.

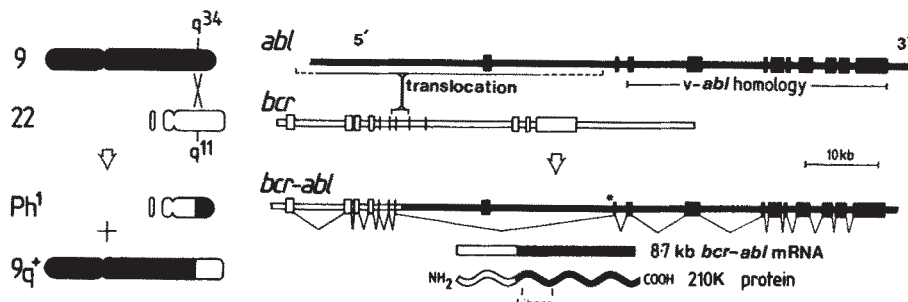
With such a gap between the *bcr* and *abl* sequences, how can a fused *bcr-abl* messenger RNA, and hence polypeptide, be made? It is assumed that a giant primary transcript is made from the entire *bcr-abl* locus and Shtivelman *et al.* plausibly suggest that, by means of the subsequent RNA splicing, the last *bcr* exon before the fusion point always becomes joined to the second *abl* exon (marked with an asterisk in the figure) in the messenger RNA. In this scheme, the first *abl* exon (hatched) will be lost during RNA splicing because it lacks the 'splice acceptor' signal required for splicing to the *bcr* exon. Consequently, in each CML patient there will be a novel mRNA of about 8.7 kb that includes precisely the same *abl* mRNA segment, despite the large variation in chromosome 9 breakpoints. In support of this model, cells from two CML patients with different chromosome 9 breakpoints generate *bcr-abl* mRNAs in which the *abl* segment

begins at precisely the same nucleotide¹⁰.

Cells from all CML patients so far examined contain an *abl* polypeptide of relative molecular mass of approximately 210,000 (210K), whereas normal cells contain only a 145K *abl* polypeptide^{8,9}; the N-terminal segment of the normal *abl* polypeptide, probably comprising at least 25 amino-acid residues, has been replaced by some 600-700 residues from the N-terminus of the *bcr* product. Since all the *bcr* breakpoints seem to fall after the second or third of several very small *bcr* exons, the predicted *bcr-abl* polypeptides may differ only by the presence or absence of the third small *bcr* exon, which encodes 24 amino-acid residues¹¹. It is intriguing to note that the *v-abl* polypeptide encoded by the Abelson virus is also an N-terminal substitution of *c-abl*; 236 amino acids of the viral *gag* polypeptide replace about 113 residues of the normal cellular *abl* product. Hence N-terminal substitution may be critical to convert *c-abl* to a true oncogene.

How the *bcr-abl* gene may promote myeloid cell proliferation is unclear, but one interesting functional difference between it and *c-abl* has already emerged. In common with the *v-abl* gene product, the 210K polypeptide from CML cells has tyrosine kinase activity; that is, it catalyses the transfer of a phosphate from ATP to tyrosine residues in certain proteins^{8,9}. At least as assessed by autophosphorylation, the product of normal *c-abl* lacks kinase activity^{8,9}, although it will probably be found when the appropriate substrates are identified, since *c-abl* bears a tyrosine kinase domain. Thus, it seems likely that the substrate specificity of *c-abl* is altered by the replacement of its N-terminus by *bcr* sequences. Perhaps that modification also explains why the *bcr-abl* gene is most effective in transforming myeloid cells whereas *v-abl* preferentially transforms early B lymphoid cells¹².

Receptors for several growth factors, including insulin and epidermal growth factor, are tyrosine kinases, so it is possible that the normal *c-abl* product is a com-



Generation of the Ph^1 chromosome by recombination between the *abl* locus at band q34 of the chromosome 9 and the *bcr* locus at q11 of chromosome 22. The reciprocal 9;22 translocation is depicted on the left and the corresponding gene structures on the right, with boxes and lines denoting the approximate position of large and small exons, respectively. In the *abl* locus, the position and size of the 5' exons is not yet well fixed, nor have the breakpoints been located in most instances, although most seem to be a considerable distance 5' to the *abl* gene. For *bcr*, not all exons have yet been defined but all breakpoints fall within the bracketed region. In the *bcr-abl* locus, a nuclear precursor RNA (thin lines) is presumed to be spliced in such a way that sequences of the most 5' *abl* exon (hatched) are excised during generation of the illustrated 8.7 kb hybrid *bcr-abl* mRNA, which almost certainly encodes the 210K polypeptide that contains the *abl* tyrosine kinase domain at the indicated region. The molecular counterpart of the 9q⁺ chromosome is not shown.

ponent of a growth factor receptor. On the Ph¹ chromosome, the expression of the fused *bcr-abl* gene is presumably regulated by the control elements of the *bcr* gene. Since this chromosomal abnormality features prominently only in CML, which is a neoplastic proliferation of haematopoietic stem cells, it is tempting to speculate that the normal *bcr* gene is expressed primarily in that cell lineage. If the *bcr* product proves to be a cell-surface protein, one intriguing possibility would be that *bcr-abl* represents a hybrid receptor.

While the function of *bcr-abl* is a matter of speculation, there now seems little reason to doubt that its formation by the 9;22 translocation is a critical step in the development of CML. Thus the work on the Ph¹ chromosome, when considered together with the large body of work on *myc* oncogene translocations in lymphoid

tumours, makes a strong case that many of the karyotypic alterations found in neoplastic cells represent the activation of specific cellular oncogenes. □

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Neurobiology

Orchestration at the synapse

from Clarke R. Slater

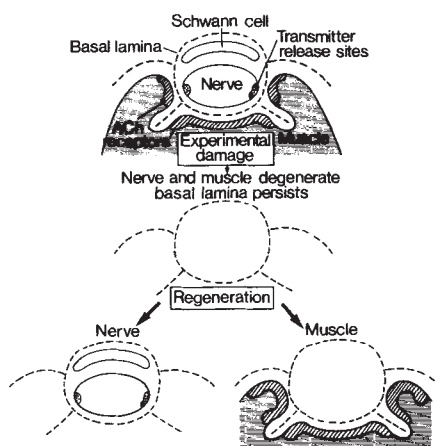
CHEMICAL transmission between neurones or from neurone to muscle takes place at junctions termed synapses. Structural specializations at these junctions ensure that receptor proteins in the post-synaptic membrane are aligned opposite the sites in the presynaptic membrane where transmitter is released. How this alignment comes about and is maintained is important for understanding the hardware of the nervous system and its development. Recent work from U.J. McMahan and his colleagues on the neuromuscular junction has shown that the basal lamina is important for determining the distribution of synaptic components on both sides of the synapse^{1,2}. In two papers starting on page 571 of this issue, McMahan *et al.* now show that the synaptic basal lamina contains a component chemically very similar to one that causes clustering of molecules that are essential for the functions of the neuromuscular junction^{3,4}.

The basal lamina is an extracellular layer of material composed largely of a form of the durable connective tissue protein, collagen. A basal lamina sheath surrounds each skeletal muscle fibre and each motor nerve fibre. At the neuromuscular junction, nerve and muscle share a common layer of basal lamina, which has been known for some time to differ chemically from other basal laminae in that it contains a high concentration of acetylcholinesterase (AChE), the enzyme that breaks down acetylcholine (ACh), the transmitter chemical at the neuromuscular junction.

The idea that the basal lamina might influence the distribution of other synaptic components emerged from a series of experiments in which the cells of the neuromuscular junction were destroyed and then either the nerve or the muscle cell

was allowed to grow back within the framework provided by the persisting basal lamina sheaths (see figure). When the regenerating nerve made contact with the original synaptic basal lamina, detectable by its persisting AChE activity, it stopped growing and formed the structural features characterizing transmitter release sites¹. When the muscle cell made contact with the synaptic basal lamina, a high density of receptors for ACh formed in its surface membrane at the site of contact². As no cellular components of the original neuromuscular junction persisted to induce the local specialization of the regenerating cells, it seems that this information could only have come from the surviving basal lamina.

Since those experiments were reported, several groups have been trying to identify molecules that might mediate the effect of synaptic basal lamina on the distribution of ACh receptors. One approach has led



Summary of experiments indicating that the basal lamina regulates the distribution of synaptic components (adapted from ref. 2).

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to the isolation of a variety of tissue extracts that cause the aggregation of ACh receptors on cultured muscle cells. In other studies, antibodies that specifically label synaptic basal lamina have been raised and used to isolate and characterize the antigens. McMahan *et al.* have effectively combined these two approaches. Starting with a preparation of basal lamina from the electric organ — essentially a stack of giant neuromuscular junctions — of the fish *Torpedo*, they obtained a soluble extract that increased the clustering of ACh receptors on cultured chick muscle cells when present at a concentration of not more than 10^{-10} M. An antiserum against this extract bound specifically to the synaptic basal lamina of frog neuromuscular junction and blocked the ability of the extract to induce clusters of ACh receptors⁵.

In the first of their present papers, McMahan *et al.* describe monoclonal antibodies, raised against the same extract, that have effects similar to those of their original antiserum³. In the second paper⁴, they report that the extract causes the formation of clusters of AChE activity, as well as clusters of ACh receptors. It is very likely that both clusters occur at the same sites. Because of the specificity and homogeneity of monoclonal antibodies, it can be concluded that a component of the basal lamina at the neuromuscular junction is closely related to, if not identical with, molecules that can induce the clustering of the two most essential molecular components of the synaptic surface of skeletal muscle cells. By studying the effect of these antibodies on the developing neuromuscular junction, it should be possible to define the role that the basal lamina plays in synapse formation.

The antibodies will also be useful in exploring whether the cluster-inducing molecules are present in the central nervous system and how they are produced during synapse formation. Recently, Kelly and his colleagues described a glycoprotein component of the basal lamina of the electric organ⁶. This molecule is also present in the spinal cord of *Torpedo*, where it is located specifically in the electromotor neurones and is transported in their axons to the periphery. This raises the possibility that, during development, growing motor nerves add components to the basal lamina of the muscle fibre that cause aggregation of the molecules constituting an effective synapse. Experimental tests of such hypotheses should soon be possible. □

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Palaeontology

Cosesaurus — the last proavian?

from Andrew R. Milner

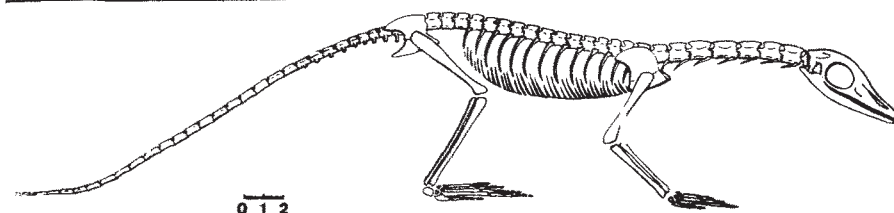
SYSTEMATIC palaeontology sometimes seems to resemble a scene from Dante's Purgatory. As fast as some palaeontologists tidy fossils into manageable groups, others describe new specimens which cannot possibly fit into any orthodox framework. Some supposedly bizarre or highly significant fossils do maintain their status on investigation but it has been, and still is, the fate of many an oddity to prove on revision to be more mundane than its original author(s) believed. One such fossil, *Cosesaurus aviceps*, now seems to have joined the ranks of the demoted.

Cosesaurus was first described a decade ago as a proavian from the Middle Triassic of Spain¹. It is a small fossil reptile, 14 cm long, represented only by a single negative impression of a skeleton having a large-eyed skull with a relatively large brain case and beak-like jaws, a lizard-like body with a long tail and hindlimbs slightly longer than the forelimbs. It was originally described as possessing a furcula (the 'wish-bone', or fused clavicles), a bird-like pelvis and traces of feather impressions; this evidence, coupled with the bird-like skull, led one of its discoverers to propose it as a closer relative of true birds than *Archaeopteryx*². *Cosesaurus* was even used to support the hypothesis of a Palaeozoic origin for the birds, quite distinct from dinosaurs or even archosaurs³. It seemed in many respects to be the much-sought arboreal 'proavis' rendered tangible at last. But *Cosesaurus* never gained many adherents as a protobird and, in recent years, the relationship of *Archaeopteryx* and the birds to the dinosaurs has been thoroughly supported by cladistic analyses^{4,5}. This has left *Cosesaurus* in an increasingly anomalous position.

A recent reassessment by Sanz and López-Martínez seems to have found a more modest position for *Cosesaurus* in the phylogenetic framework of the sauropsid reptiles⁶. They dismiss the supposed furcula and feathers as overinterpretation of a poor specimen, and argue that the remaining suite of derived characters support a position within the Prolacertiformes, a group of Permo-Triassic diapsid reptiles, once thought to be early lepidosaurs, but more recently argued to be primitive relatives of the archosaurs^{7,8}. Sanz and López-Martínez also note *Cosesaurus*'s specific resemblance to the much larger *Macrocnemus*, a prolacertiform from the contemporary and ecologically equivalent Monte San Giorgio in Switzerland site (see figure). They suggest that other characteristics, including the superficially bird-like head, are juvenile: if *Cosesaurus* is related to *Macrocnemus*, it must be either a hatchling or a dwarf species, the former being more

likely in the absence of other evidence.

This identification agrees well with the presence of the nothosaur genera *Lariosaurus* and *Nothosaurus* in the same beds at both localities and implies that the Spanish site has simply produced a small



Reconstruction of *Macrocnemus bassanii* from two specimens from the Monte San Giorgio site, Switzerland, studied by B. Pryer. The limb bones are shown in their longest view, without an anatomical perspective. Scale in cm. Drawing by N. López-Martínez (from ref. 6).

sample of the Monte San Giorgio-Muschelkalk vertebrate association — a typical Middle Triassic Tethys coastal fauna. The general resemblance between the skull of *Cosesaurus*, a juvenile archosauriform, and that of a bird is an interesting reminder of how progenetic dwarfing may have reshaped the typical archosaur skull to result in the avian skull.

Another strange reptile recently and more cautiously described from the Upper Triassic of Italy⁹ is less likely to suffer such a reduction in status. *Megalancosaurus preonensis* is also tiny and has a superficially bird-like head, long cervical vertebrae, a very long forelimb with five short digits, each bearing a deep blade-like claw, slender ribs and an archosaur-type

pelvis. Only a preliminary description has yet been published and only a tentative position within the thecodont-grade archosaurs has been proposed.

Some recent analyses of archosaur relationships place the pterosaurs and dinosaurs close together within the archosaurs¹⁰ and, judging by such characteristics as are visible, *Megalancosaurus* seems to belong close to, or within, the pterosaur-dinosaur clade. Its small size, peculiar claws and slender forelimbs suggested to its describers that it

was arboreal and, indeed, its forelimb is distinctly reminiscent of that of the Colugo or flying lemur, the patagial gliding mammal of south-east Asian forests. Could it be that this fossil represents a conservative patagial gliding relative of the pterosaurs?

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Geochemistry

Uses for synthetic fluid inclusions in quartz crystals

from Edwin Roedder

FLUID inclusions are small droplets of the fluids from which crystals grew and are found in many minerals, both natural and synthetic. In natural gem minerals, fluid inclusions diminish their clarity, and hence their value; in synthetic crystals they detract from the useful mechanical, electrical or optical properties for which the crystals are grown. Why then should anyone want purposely to form microscopic inclusions of fluid in otherwise optically clear quartz crystals? Yet S.M. Sterner and R.J. Bodnar¹ have recently developed a procedure to do just that, and have shown that the resulting inclusions are valuable in a surprisingly wide range of geochemical and mineralogical studies that may help us to understand better the natural processes that may have taken place millions of years ago.

Sterner and Bodnar start with solid cylinders of clear quartz, cut from a fluid-inclusion-free natural crystal. These are heated and then cracked by quenching in water. The cracked but still intact cylinders are dried and sealed into thin-walled noble-metal capsules with the starting materials appropriate to generate the desired fluid composition under the experimental conditions. The collapsible capsules are then subjected to external pressures of 0.5–7 kbar, while being heated to 200–850°C, for periods of between 3 hours and 87 days, before being quenched.

During the time at elevated temperature and pressure, the fractures in the quartz crystal are healed by dissolution and recrystallization of the crack surfaces, thereby decreasing the total surface energy

(see Fig. 1). In so doing, small portions of the capsule fluid are trapped within the new growth — samples of the fluid, sealed in single crystal quartz 'bottles'. Thus, these new synthetic inclusions are analogous to the 'secondary' inclusions found in most natural quartz crystals (Fig. 2). (Secondary inclusions are those that are formed by the healing of fractures in a pre-existing crystal; the fractures are generally caused by external stress.) Possible applications include the study of inclusion-trapping mechanisms and rates, post-trapping changes and even the determination of phase equilibria and pressure, volume and temperature data on otherwise experimentally very difficult high-temperature, high-pressure hydrothermal systems.

The full range of uses for these synthetic fluid inclusions has yet to be explored but applications already discussed by Sterner and Bodnar¹ have some fascinating ramifications. Fluid inclusions, both natural and synthetic, undergo various phase changes on cooling after becoming enclosed in their crystal host. Henry Clifton Sorby (the 'father of fluid inclusions') showed in a study 127 years ago² that these phase changes could be observed and reversed in the laboratory while heating the host crystal under the microscope. The most common phase change observed occurs because liquids shrink at a much higher rate than crystals. Thus, if a hot liquid is sealed into a relatively rigid, fixed-volume crystal 'bottle' and then cooled, differential shrinkage of the liquid relative to the crystal will cause the formation of a vapour bubble in the liquid. Sorby observed the point when this bubble disappeared in a crystal on a microscope heating stage and used it as a measure of the temperature at which the cavity had originally been sealed, perhaps millions or

billions of years earlier. Each tiny inclusion (and ordinary white quartz may contain 10^9 inclusions per cm^3) is, in effect, a built-in thermometer, recording the temperature of a past event.

Sorby's proposal to use fluid inclusions in this manner — only one of his many brilliant ideas — was, and still is, the subject of considerable discussion. In part this debate arose because the geothermometric data from fluid inclusions contradicted some of the preconceived notions of the day, and in part because of the difficulty in assuring that the geological conditions under which an inclusion formed actually fulfilled the several relatively simple but necessary assumptions behind the method. Previous studies of fluid inclusions accidentally trapped in synthetic industrial quartz crystals, grown under approximately known conditions, have verified the general validity of the concept of inclusion geothermometry. Several minor problems have, however, interfered with the widespread use of the method, not the least of these being, surprisingly, that of accurately calibrating the microscope heating stages³. The most common application of synthetic inclusions made with the new method may be as a device for calibrating instruments.

In the years since Sorby's work, fluid inclusions have provided much geological information in addition to temperature. Measurements of phase changes visible under the microscope at temperatures ranging from roughly -190°C upwards, and with appropriate conditions and caveats, can provide data on the density, pressure and the major solute and solvent compositions of the fluids at the time of trapping. Most of these data simply cannot be obtained from any other source. In the search for metallic ore deposits, it would obviously be desirable to understand the chemical processes that resulted in transport of the ore metal to the site of the ore body, and its precipitation there. This requires an understanding of the chemistry of the ore-forming fluid; with a few rare exceptions, the only samples we have of these fluids are in microscopic fluid inclusions.

The past few decades have seen a tremendous increase in studies of the composition of such inclusions. Many of these have involved the use of new methods of ultra-microchemical analysis, such as laser-activated micro-Raman spectroscopy and laser-ionized mass spectrometry. Such methods have attempted to overcome the difficult problems of extraction and chemical (or isotope) analysis of major and trace constituents of these inclusions, without serious loss or contamination. In addition to the exceedingly small total sample size (frequently less than 10^{-9}g) usually available, the problem of finding suitable analytical standards has been particularly limiting. The new synthetic inclusion technique developed by Sterner and Bodnar permits the preparation of optimum standards for both microscopy and chemical analysis — inclusions of the same

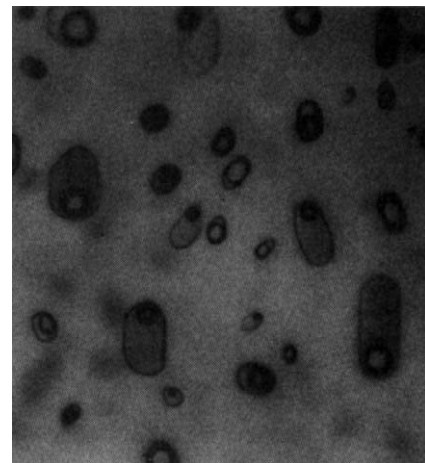


Fig. 2. Synthetic fluid inclusions in quartz. Those with small bubbles (of CH_4) initially trapped liquid and are now dominated by a carbonate; those with large bubbles trapped vapour and are largely CO_2 ; $\times 156$ (photomicrograph courtesy of S.M. Sterner and R.J. Bodnar).

size range, enclosed in the same host mineral and containing fluids of known density and composition.

Still another application, with a particularly exciting potential, is the use of these synthetic inclusions in studies of the physical chemistry of otherwise difficult chemical systems. N.P. Ermakov⁴ pointed out 35 years ago that fluid inclusions in transparent minerals are unique miniature 'visual autoclaves', permitting visual observations of high-temperature and high-pressure phase assemblages and critical phenomena. Bodnar and Sterner⁵, have explored the use of synthetic inclusions in pressure-volume-temperature studies of hydrothermal systems and Bodnar *et al.*⁶ have gone on to show how valuable these synthetic inclusions can be in the determination of the phase-equilibrium properties of the system $\text{H}_2\text{O}-\text{NaCl}$ to $1,000^\circ\text{C}$ and 1,500 bar. Such data are necessary for an understanding of several important groups of ore deposits that have formed from hot, extremely saline fluids and are also useful in understanding the mechanism and energetics of volcanic eruptions such as that at Mount St Helens in May 1980. Conventional experimentation is very difficult because these fluids are exceedingly corrosive. The use of even a few trial runs with synthetic inclusions to locate the critical fluid parameters could reduce the effort involved in larger-scale experiments, or the inclusion technique itself could even be used for the entire phase-equilibrium study. □

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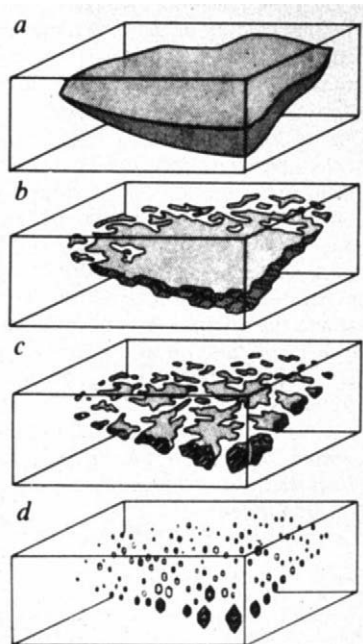


Fig. 1. Stages in the healing or 'necking down' of a crack in a quartz crystal resulting in secondary negative crystal inclusions (from ref. 3).

Oncogenes homologous to steroid receptors?

SIR — Why do tumours that initially respond to hormone therapy lose their hormone responsiveness after a time and then grow autonomously? This problem is of great clinical importance, for instance in breast cancer where tumours that diminish in size after endocrine treatment almost invariably recur and then tend to progressively become nonresponsive. The discovery that many breast cancers consist of subclones of oestrogen receptor (ER)-positive and ER-negative cells^{1,2} suggests that tumour progression is the survival of the 'fittest' and most malignant ER-negative subtype. But where do the hormone-independent cells come from in the first place? Are they always present or are they derived from hormone-dependent cells? And how do these manage to survive and proliferate if they do not have the oestrogen receptor to give the proliferation signal?

A general opinion is that the cells become hormone-independent because they have *lost* the receptor. But such cells should be unable to respond to their growth stimulus and so be eliminated from the tumour population. A more plausible alternative is that they have undergone a change that leads to their getting a growth signal even in the absence of ER. Steroid receptors interact with nuclear DNA at enhancer regions that regulate the transcriptional activity of structural genes³⁻⁶. Perhaps a mutation in the enhancer leads to its activation of genes without the need for receptor to bind to it. However, it is unlikely that the loss of ER and a mutation in the enhancer region would occur simultaneously. We suggest another explanation, namely the appearance of aberrant receptor-like molecules that bind to DNA and give a proliferation signal even in the absence of oestrogen. Unlike traditional ligand-binding studies, the recently developed monoclonal antibodies to steroid receptors have made it possible to seek aberrant receptors which fail to bind steroids in hormone-independent cells. Thus immunoreactive glucocorticoid receptors have been detected in receptor deficient (r^-), nuclear-transfer deficient (nt^-) and nuclear-transfer increased (nt^+) mouse lymphoma variant cells⁷, and this has been confirmed by the finding that mRNA encoded by the receptor gene in these variants is changed quantitatively and qualitatively⁸. In r^- cells, one allele is altered so that no product is detectable with either steroid or antibody, while the other allele produces an immunoreactive polypeptide devoid of hormone-binding activity⁷. In nt^- and nt^+ lymphoma cell variants, the glucocorticoid receptors react abnormally with DNA^{9,10}.

What are the structural changes associated with this abnormal functioning of receptors? In nt^+ cells part of the recep-

tor domain is missing which binds neither steroid nor DNA, but which may modulate nuclear association and DNA binding^{7,11}. The complexes of this aberrant receptor plus glucocorticoid might still bind to the biologically relevant sites on DNA, but without this leading to transcriptional regulation⁷. Thus, the available evidence suggests that the loss of steroid responsiveness of some tumour cells may be due to alterations in the steroid receptor gene(s) leading to altered receptor molecules which may act in a 'deregulated' manner and induce cellular proliferation even in the absence of hormone. The mutated (possibly truncated) receptors may or may not retain the hormone binding properties. A biological precedent for this possibility has been described: the *v-erb-B* oncogene product is homologous to the non-hormone-binding domain of the epidermal growth factor (EGF) receptor and cells expressing the gene do not require EGF for continued growth¹². This raises the question: are there oncogenes that are homologous to parts of the steroid receptor genes and confer loss of hormone dependence upon cells that contain them? If so, is this because the aberrant receptors produced by these oncogenes have the ability to bind to DNA without the aid of steroid hormones, and so give a continuous growth signal to the cell? In this area of research progress is rapid, so we should soon know the answer to these important questions.

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The infectious nature of prions

SIR — In a recent discussion of prions, Colin Masters emphasized that a protein-only composition of prions requires protein-directed protein synthesis for its replication, "a situation which could be without precedent in biological science"¹. While protein-directed protein synthesis may be without precedent, protein-directed protein-folding has a precedent and could explain the infectious nature of prions.

Geneticists are well aware of this as

a mechanism of gene effect in a number of dominantly inherited diseases. For instance, it is possible for an abnormal collagen to cause a severe phenotypic abnormality², while a non-expressing mutant for collagen is recessive³.

This is most easily interpreted if the mutant collagen gene directs the synthesis of abnormal collagen chains which affect structure in a negative way through mixed trimers with the normal collagen chain which are conformationally abnormal. One can also see the effects of the conformation of one protein chain on another in haemoglobins (for instance, the increased affinity of a normal alpha-chain for oxygen secondary to a mutant beta-chain such as occurs with haemoglobin Ypsilanti⁴).

These protein interaction examples involve inherited genes, but the interaction is not transmitted, that is the original information is in the genome but the negative effects of an altered protein conformation are limited to the zygote. However, it is possible for such altered conformational information to be transmitted from generation to generation. The best example I know of this is that of reversed cortical pattern in *Paramecium*. Beisson and Sonneborn showed some years ago that a reversed patch of cortex could be transmitted to descendant *Paramecium* through its usual binary fission⁵. Thus, the cortex grows by the addition of macromolecules to a pre-existing cortex, receiving positional information from the pre-existing cortex. When the positional information is altered in some way, in this case by microsurgery, the abnormal information can be transmitted.

It is conceivable that the prion could be a normal protein in an abnormal configuration which, upon transmission to a new host, leads the normally synthesized protein to start folding in the new abnormal configuration, thus multiplying the number of prions in the abnormal conformation. In order to account for the increase in the number of prions, one must assume that the prion is not permanently affixed to the aggregate of 'new' and 'old' prions and that the new configuration of this normally-present protein is highly stable. Of course, such a hypothesis does not help us understand how prions cross cell membranes and the other aspects of infectivity which must be taken into account to explain their transmission between individuals.

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High-precision timing observations of the millisecond pulsar PSR1937+21

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Long-term studies of pulse arrival times for the pulsar PSR1937+21 yield estimates of the pulsar's period, spin-down rate and celestial coordinates with unprecedented accuracies. Over periods exceeding six months, this pulsar is at least comparable in stability to the best man-made atomic clocks. Our results place a limit on the energy density of low-frequency gravitational waves in the Universe.

SHORTLY after the discovery¹ of PSR1937+21, we began a series of high-precision measurements of the arrival times of its pulses, observations being made at intervals ranging from daily to once every several weeks since November 1982. Data collected in the first two weeks were sufficient to determine the period derivative of the pulsar². Here, we present an analysis of a much longer span of data obtained between 28 November 1982 and 12 October 1984. The results confirm our earlier measurements of the pulsar position, period and spin-down rate, improving their accuracies by several orders of magnitude.

The microwave beam that produces the pulses from PSR1937+21 has a constant angular width of $\sim 15^\circ$ and is rigidly attached to a spinning neutron star with rotational kinetic energy of almost 10^{52} erg. The measured rate of energy loss, presumably resulting from low-frequency electromagnetic radiation and particle acceleration in the pulsar magnetosphere, corresponds to a rotational quality factor, $Q > 3 \times 10^{19}$. After a constant slow-down rate has been removed from the timing data, we find that the measured fractional stability of the pulsar is better than one part in 10^{13} over timescales of ≥ 6 months. This remarkable long-term stability is equal to or exceeds that of the best existing atomic clocks.

There are many potentially interesting applications of a series of regular comparisons between a distant, natural standard of dynamical time and Earth-bound atomic clocks. For example, we already can place cosmologically interesting limits on a stochastic background of gravitational radiation in the Universe, by extending to higher precision the methods used recently with slower pulsars^{3,4}. In addition, we can use the data to achieve sub-milliarc second astrometric accuracies relative to a coordinate system based on the motion of planets in the Solar System. Consequences of the astrometric results of this experiment will be published elsewhere⁵. Over the next few years there is good reason to expect that timing data on PSR1937+21 will help to elucidate knowledge of the Earth's motion in the Solar System, provide substantially tighter limits on the energy density of low-frequency gravitational radiation in the Universe and possibly even provide a useful new long-term standard of dynamical time.

Arrival time observations

Our observations have been made at 1,408 MHz, using the Arecibo 305-m telescope and the Princeton pulsar-timing equipment⁶ developed for observations of the binary pulsar PSR1913+16. Incoming signals with both senses of circular polarization are amplified, converted to 30 MHz and introduced to a $2 \times 32 \times 0.25$ MHz filter-bank receiver. The detected outputs from the two orthogonally polarized channels at the same frequency are added, and the 32 total-power sums are sampled

at ~ 25 - μ s intervals and progressively summed in a digital delay line serving as a de-disperser⁷. Output from the de-disperser is converted back to analogue form and fed to a signal averager which samples the signal every 1/64 of a period and produces an integrated pulse profile for each 2 min of observation. The sampling is accurately synchronized to the pulsar period and corrected for Doppler shifts induced by the Earth's motion. Near the middle of each 2-min integration the time corresponding to the sample stored in the first 'bin' of the accumulating waveform is automatically recorded; at the end of each integration, the timing information is saved on magnetic tape together with the observed pulsar waveform.

Approximately 60 integrations can be recorded during the time the pulsar remains in view on a given day. After the observations are complete, the phase of each 2-min integrated profile is determined by cross-correlation with a 'standard profile' formed by averaging a large number of profiles. To reduce the volume of data, we then average the observed phases in groups of 15 consecutive integrations. The mean phase is converted to seconds and added to the time recorded for the first bin at the midpoint of the eighth integration, thereby yielding an effective pulse arrival time for each 30 min of data.

Our basic observational data now consist of 196 30-min observations of pulse arrival time at the Arecibo telescope, measured in a locally kept approximation to coordinated universal time (UT). During these observations, time at Arecibo Observatory was maintained by a clock that counts the cycles of a 5-MHz signal from a Hewlett-Packard model 5641 rubidium clock. The offset between this clock and UT (kept by the United States Naval Observatory, USNO) is measured once a day by the use of Loran-C timing signals broadcast from Jupiter, Florida. (The free-running rubidium clock typically accumulates phase drifts of a few tenths of a microsecond per day.) We apply retroactive corrections to epochs of the Loran-C signals, as published by USNO, to remove drifts of the master clock in Florida relative to the more accurate master clock kept by USNO in Washington. In addition, epoch comparisons with other time standards are made occasionally by means of very long baseline interferometry observations⁸ and by occasional checks with travelling clocks from USNO. We believe that our corrected timescale at Arecibo Observatory has been consistent with UT (USNO) to within 1 μ s at most times.

Data analysis

Our goal in analysing the data of this experiment is to compare the pulsar 'clock' with a uniform standard of dynamical time. To this end, we convert our measured times to UT (USNO) and then to International Atomic Time (TAI) by adding a constant offset plus appropriate leap seconds. The topocentric TAI arrival times are converted to times at the Solar System barycentre by applying geometry based on interpolations of an accurate ephemeris of the Earth's motion. We also adjust the arrival times to correct for: (1) dispersive delays in interstellar and

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interplanetary space; (2) relativistic propagation delay caused by the pulsar signal moving through the gravitational field of the Sun; (3) gravitational redshift in the rates of terrestrial clocks caused by the changing gravitational potential at Earth; and (4) varying time dilation caused by changes in the Earth's velocity. Our procedure is basically that described by Manchester and Taylor⁹ and used routinely for slower pulsars^{10,11}. However, the precision desired in the present experiment requires an improved model for the conversion of proper time at Washington, TAI (USNO), to coordinate time at the Solar System barycentre, or barycentric dynamical time (TDB).

Corrections applied to the observed pulse arrival times are summarized in the equation

$$t_b = t_{\text{obs}} + (\mathbf{r} \cdot \hat{\mathbf{n}})/c + \Delta t_r - \Delta t_g - D/f^2 \quad (1)$$

where t_b is the infinite-frequency coordinate time of pulse arrival at the Solar System barycentre, t_{obs} is the measured TAI of pulse arrival at the observatory, $\mathbf{r}$ is the vector displacement of the telescope from the barycentre, $\hat{\mathbf{n}}$ is a unit vector pointing from the barycentre to the pulsar, c is the speed of light, Δt_r is the relativistic correction from proper time (TAI) to coordinate time (TDB), Δt_g is the gravitational propagation delay, D is the dispersion constant and f the observing frequency, transformed to the barycentric reference frame. Note that for the convenience of terrestrial observers, our unit of time, even for quantities measured in the barycentric frame, is the A.1 second as measured by a fictitious clock moving in a circular orbit 1 AU from the Sun. (This timescale is not presently defined by any international agreement. A precise definition must also specify an interval over which the gravitational influences of the planets have been averaged; for the proper times-to-coordinate times tabulated in the PEP740-R ephemeris (J. F. Chandler *et al.*, personal communication), this interval was JED 2430000–2451545.)

In practice, we obtain the required values of $\mathbf{r}$ and Δt_r by interpolation of the Center for Astrophysics PEP740-R ephemeris, using 10-point interpolation formulae. Calculation of Δt_r by numerical integration of the planetary ephemerides is necessary to achieve the desired accuracy of 0.1 μs . (The closed-form approximation derived by Moyer¹² seems to contain errors of a few μs .) After applying all known corrections of $\geq 0.1 \mu\text{s}$ amplitude to obtain values of t_{obs} in TAI units, and then calculating t_b with the aid of equation (1), we obtain a series of barycentric arrival times t_i , $i = 0, 1, \dots, 195$, where we have dropped the original subscript on t_b . The expected pulsar phase (in turns) corresponding to the i th arrival time is given by

$$\phi(t_i) = \phi_0 + \nu_0(t_i - t_0) + \frac{1}{2}\dot{\nu}(t_i - t_0)^2 \quad (2)$$

where ϕ_0 , ν_0 and $\dot{\nu}$ are the pulsar phase, frequency and frequency derivative, respectively at time t_0 . The arrival time 'residual' (in s) is then given by

$$R(t_i) = \frac{\phi(t_i) - n(t_i)}{\nu_0} \quad (3)$$

where $n(t_i)$ is the integer closest to $\phi(t_i)$. Using standard procedures we minimize the sum-of-the-squares of all residuals, solving for corrections to ϕ_0 , ν_0 , $\dot{\nu}$ and the two position and two proper motion terms implicit in the unit vector $\hat{\mathbf{n}}$ in equation (1). The results of such a fit are shown in Table 1, where by convention we list the pulsar period and derivative, instead of frequency and derivative. Post-fit residuals from the solution are plotted in Fig. 1.

It is clear from Fig. 1 that the residuals are dominated by systematic effects, rather than by random measurement errors. For example, residuals from data obtained in a single day tend to be highly correlated and weaker correlations over timescales of several months are also evident. Possible causes of systematic errors that we cannot yet eliminate at the $\sim 1 \mu\text{s}$ level include clock errors (perhaps related to the transfer of time from Washington to Arecibo), imperfect removal of dispersion from the scintillating pulsar signal, ephemeris inaccuracies and small changes in propagation time caused by interstellar gas clouds

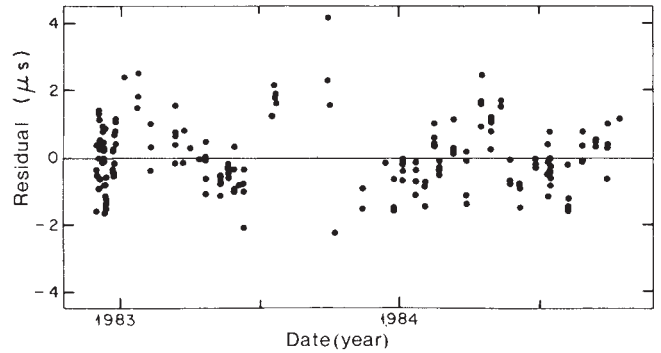


Fig. 1 Post-fit timing residuals for PSR1937+21, after a solution for period, period derivative, position and proper motion. Each circle represents a 30-min observation; the r.m.s. residual is 0.9 μs .

Table 1 Measured parameters of PSR1937+21

Period	$P = 0.0015578064488724 \pm 2 \text{ s}$
Period derivative	$\dot{P} = (1.05110 \pm 0.00008) \times 10^{-19} \text{ s s}^{-1}$
Right ascension (1950.0)	$\alpha = 19 \text{ h } 37 \text{ min } 28.74600 \text{ s} \pm 0.00003 \text{ s}$
Declination (1950.0)	$\delta = 21 \text{ h } 28 \text{ min } 01.4606 \text{ s} \pm 0.0010 \text{ s}$
Proper motion in α	$\mu_\alpha = -0.3 \pm 0.4 \text{ marc s yr}^{-1}$
Proper motion in δ	$\mu_\delta = -1.4 \pm 1.0 \text{ marc s yr}^{-1}$
Epoch (at barycentre)	$t_0 = 2445303.2731658 \text{ JED}$
Assumed dispersion	$D = 2.94788 \times 10^{17} \text{ Hz}$

Quoted positions are in the coordinate system of ephemeris PEP740-R.

drifting across the line-of-sight¹³. Since October 1984 we have been making observations designed to identify and reduce these possible sources of error. (Time is now transferred to Arecibo Observatory by signals from satellites in the Global Positioning System, with accuracies in the tens-of-nanoseconds range. In addition, we have built a new multichannel signal averager that exactly follows the inverse frequency-squared dispersion law.)

For the 1982–84 data, however, we must conclude that the root-mean-square residual, $\sim 0.9 \mu\text{s}$, is dominated not by instabilities in the pulsar or by receiver noise but by systematic (possibly instrumental) effects. For this reason, we have adopted a conservative attitude toward the formal standard deviations of the parameter estimates. The uncertainties quoted in Table 1 are four times the formal standard errors from the least-squares fit, and are approximately equal to those which would be obtained if we had available one measurement of $\sim 2 \mu\text{s}$ accuracy for each observing day. We hope to be able to relax this cautious procedure in the near future and to improve further the accuracies of the fitted parameters.

Pulsar parameters

The pulsar period and derivative listed in Table 1 are consistent with, but much more accurate than, our previously published values². They confirm that the characteristic age of this pulsar is rather large, $P/2\dot{P} \approx 2 \times 10^8 \text{ yr}$, and that if the slow-down torque is provided by magnetic dipole radiation, the surface magnetic field must be unusually small, $3.2 \times 10^{19} (P\dot{P})^{1/2} \approx 5 \times 10^8 \text{ G}$ (where G is the Newtonian constant) (see, for example, Chapter 9 of ref. 9). Attempts to solve for a second derivative of period yield only an upper limit, $|\ddot{P}| < 10^{-29} \text{ s}^{-1}$. This limit is not surprising because the value expected for magnetic dipole braking is $\ddot{P} = -\dot{P}^2/P \approx -7 \times 10^{-36} \text{ s}^{-1}$.

The measurement of the pulsar position in the sky described here is also consistent with previous work, but the precision of our new result is considerably higher. The uncertainties quoted in Table 1 are relative to the coordinate system implicitly defined by the PEP740-R ephemeris. The orientation of the ephemeris coordinate system relative to, say, the FK4 system, is *a priori* uncertain by $\sim 0.2''$ (J. F. Chandler *et al.*, personal communication). Recently, it has been shown that the reference frame of the PEP311 ephemeris, which is in close alignment with PEP740-R, is offset in the direction of PSR1937+21 by $\sim 0.2''$ with respect to both the Jet Propulsion Laboratory planetary ephemerides

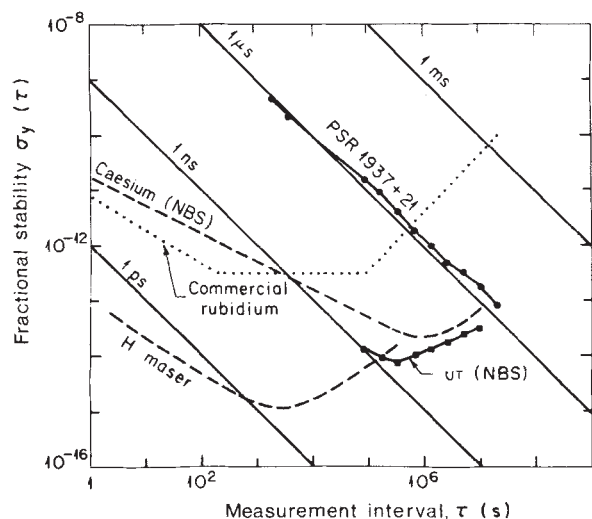


Fig. 2 Measured frequency stability $\sigma_y(\tau)$, for PSR1937+21, over intervals, τ , from 30 min to 256 days. For comparison we also plot stability curves representative of several types of atomic clocks. The curve labelled 'rubidium' refers to typical commercially available standards; those labelled 'caesium (NBS)' and UT(NBS) are based on Fig. 3 of ref. 28 and the 'H maser' curve is adapted from Fig. 5 of ref. 29.

and the FK4 system^{14,15}. These astrometric details are of considerable interest, and will be discussed elsewhere⁵.

Until we have removed all $\sim 1\text{-}\mu\text{s}$ systematic errors from the data and acquired a better understanding of possible ephemeris errors at the same level, our solution for proper motion should be treated as an upper limit of $|\mu| \leq 3 \text{ mas yr}^{-1}$. At a distance¹⁶ of 5 kpc, this limit corresponds to a transverse velocity $\leq 70 \text{ km s}^{-1}$, which is puzzling as differential galactic rotation is expected to create an apparent transverse velocity of $\sim 100 \text{ km s}^{-1}$. It is possible, of course, that the pulsar's peculiar velocity happens to cancel a large portion of the transverse component of differential galactic rotation.

It is reasonable to enquire about the magnitudes of effects not included in our timing model which might influence the pulse arrival times significantly and bias some of the parameter estimates. In Table 2 we list several such possibilities, in addition to the effects that are explicitly included in the model. Our model will need further improvements if the data are to be improved by another order of magnitude.

We are aware of several other effects that may contribute significantly to the measured pulse arrival times, such as secular changes in the Doppler shifts caused by differential galactic rotation and possible changes in G . However, these effects will be stable over very long timescales, so their effect is simply to bias our estimate of the pulsar spindown rate by $\sim 1\%$ without contributing significantly to the post-fit arrival-time residuals.

Stability of the pulsar clock

The predictability of man-made clocks is customarily measured in terms of the fractional frequency stability $\sigma_y(\tau)$ measured over various time intervals τ . The square of $\sigma_y(\tau)$, termed the 'Allan variance,' is defined by

$$\sigma_y^2(\tau) = \frac{1}{2} \left\langle \left(\frac{R(t+\tau) - 2R(t) + R(t-\tau)}{\tau} \right)^2 \right\rangle \quad (4)$$

where $R(t)$ is the post-fit residual at time t and the angled brackets signify an average taken over all available triplets of residuals spaced by $\pm\tau$. We have computed Allan variances from the post-fit residuals to the PSR1937+21 timing data, and the results are plotted in Fig. 2. For comparison we have also plotted curves that correspond to the stabilities of typical rubidium and caesium clocks and hydrogen masers and for the bank of caesium clocks maintained by the United States National Bureau of Standards. We note that although $\dot{P}/P$ for PSR1937+21 and other fast pulsars far exceeds the fractional drift rates of atomic

Table 2 Contributions to observed pulse-arrival times

Cause	Model magnitude (μs)	Estimated error (μs)
Pulsar		
Spin	$3 \times 10^{13} t$	—
Spindown	$3 \times 10^4 t^2$	—
Timing noise	—	$0.01 t^{3/2}$
Propagation at 1.4 GHz		
Interstellar medium	1.5×10^5	$0.5 t$
Interplanetary medium	0.4	<0.4
Ionosphere	—	<0.04
Troposphere	—	<0.01
Clocks and corrections		
UT(AO)—UT(JUP)	1	0.5
UT(JUP)—UT(USNO)	1	0.2
UT(USNO)—TAI(USNO)	50	0.0
Errors in TAI(USNO)	—	$0.3 t$
Reduction to barycentre		
Observatory to centre of Earth	2×10^4	0.01
Earth to barycentre	4×10^8	$0.6 t$
TAI—TDB	1,600	0.1
Gravitational propagation delay	20	<0.1
Parallax of pulsar	—	0.06

For each of the named contributions to pulse arrival time, the left-hand column gives an indication of the magnitude of the effect currently included in the model and the right-hand column gives our estimate of the errors caused by having ignored it or having modelled it imperfectly. Times, t , are measured in years. AO stands for Arecibo Observatory and JUP for the Loran station at Jupiter, Florida.

clocks¹⁷, the physical process that produces P is sufficiently well understood to predict that higher-order derivatives will be much smaller for pulsars than for atomic clocks. Thus, the timescale at which the stability curve for PSR1937+21 turns upward will probably be much longer than that for the man-made clocks.

Figure 2 makes it clear that over timescales of the order of one year, radioastronomical observations of PSR1937+21 can provide a standard of time that is competitive with any other available standard. Obviously the practical attractiveness of this new basis for barycentric dynamical time will be greatly enhanced if several more millisecond pulsars are found and their timing behaviours monitored and compared.

Gravitational radiation

According to some cosmological models, a substantial fraction of the energy density, ρ_c , required to close the Universe might be expected in the form of very low-frequency gravitational radiation^{18–21}. Specific mechanisms that have been proposed include the transition of the Universe from a de Sitter inflationary phase to a radiation-dominated era²², oscillating cosmic strings^{23,24}, a quantum chromodynamic phase transition in the early Universe²⁵ and other exotica. Clearly, the eventual measurement of a cosmic background of gravitational radiation and the determination of its spectrum could play an important role in understanding the early Universe.

At some level, the apparent stability of any distant clock sending its 'ticks' to Earth by electromagnetic signals must be limited by perturbations in the spacetime metric between the clock and Earth. This fact is the basis for using pulsar timing data or spacecraft-tracking data to place limits on the energy density of a cosmic background of gravitational radiation²⁶. The basic argument is that a plane gravitational wave of dimensionless amplitude h and period τ carries energy density

$$\rho = \frac{1}{8\pi G} \left(\frac{2\pi}{\tau} \right)^2 h^2 \quad (5)$$

Such a wave would produce pulsar timing perturbations of size $\delta t \approx h\tau$ which, if large enough, would dominate the residuals in the timing data. The longest period τ that will contribute significantly to the residuals is roughly half the time span of the observations. Thus, our two years of timing data with r.m.s. residual $\delta t = 0.9 \mu\text{s}$ would seem to place an upper limit of $h \leq \delta t / \tau = 3 \times 10^{-14}$, or $\rho \leq 2 \times 10^{-35} \text{ g cm}^{-3}$, for a stochastic background of gravitational waves of period ~ 1 year. This limit is substantially below the cosmological critical density $\rho_c = 3H_0^2 / (8\pi G) = 2 \times 10^{-29} \text{ g cm}^{-3}$ for a Hubble constant $H_0 = 100 \text{ km s}^{-1} \text{ Mpc}^{-1}$.

A more rigorous treatment of the limits that can be placed on a cosmic background of gravitational radiation from pulsar-timing data has been given by Blandford *et al.*²⁷. Their method makes quantitative allowance for the fraction of timing noise power that is absorbed into fits for the parameters of Table 1, and more accurately assesses the varying sensitivity of the method to gravitational waves of different frequencies. Their result can be summarized in the equation

$$\Omega_g(f) = 7.4 \times 10^{-5} (s-1) (\beta/N)^{(s-1)} H_{100}^2 \delta t_{\mu\text{s}}^2 \quad (6)$$

where $\Omega_g(f)$ is the ratio of gravitational wave energy density (per natural logarithm frequency interval at frequency $f \approx \beta/N$) to the cosmological critical density ρ_c , s is the power law spectral slope expected for the timing residuals due to gravitational

waves, N is the length of the data span in years, β is a parameter characterizing the effective low-frequency cutoff in the sensitivity to gravitational waves, H_{100} is the Hubble constant in units of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and $\delta t_{\mu\text{s}}$ is the post-fit r.m.s. timing residual in microseconds. For cosmologically interesting mechanisms of generating gravitational waves, s is expected to lie in the range 5–7, and for $N = 2$ Blandford *et al.*²⁷ find that $\beta = 2.05$. Thus, for $H_{100} = 1$ we can now place the very firm limit $\Omega_g < 5 \times 10^{-4}$ for a stochastic background of gravitational waves in the frequency range $\sim 1\text{--}3 \text{ cycles year}^{-1}$. This limit is not yet sufficient to constrain models which use primordial cosmic strings as an aid to galaxy formation²⁴. However, the limit on Ω_g at the lowest accessible frequencies varies approximately as $\delta t_{\mu\text{s}}^2 / N^4$, so that if: (1) the apparent pulsar stability remains high; (2) instrumental improvements can reduce the contributions of random noise and systematic errors to δt ; and (3) the duration of observations N can be significantly extended, another two or even three orders of magnitude are potentially within reach.

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Fused transcript of *abl* and *bcr* genes in chronic myelogenous leukaemia

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*Human chronic myelogenous leukaemia is characterized by a reciprocal translocation between chromosomes 9 and 22 resulting in an abbreviated form of chromosome 22 and the transfer of the *abl* cellular oncogene from chromosome 9 into the *bcr* gene of chromosome 22. Characterization of an 8-kilobase RNA specific to chronic myelogenous leukaemia shows it to be a fused transcript of the two genes. The fused protein that would be produced is probably involved in the malignant process.*

CHRONIC myelogenous leukaemia (CML) is characterized by the proliferation and accumulation of myeloid cells and their precursors¹ and is clinically divided into two phases. The initial chronic phase lasts for three to four years and the primary abnormality is a marked increase in the stem-cell compartment committed to myelopoiesis. This is followed by the acute phase (blast crisis) of 3–6 months' duration and characterized by a decrease in the differentiation ability of the cells and the loss of response to regulatory factors. In some patients there is a transitional accelerated phase.

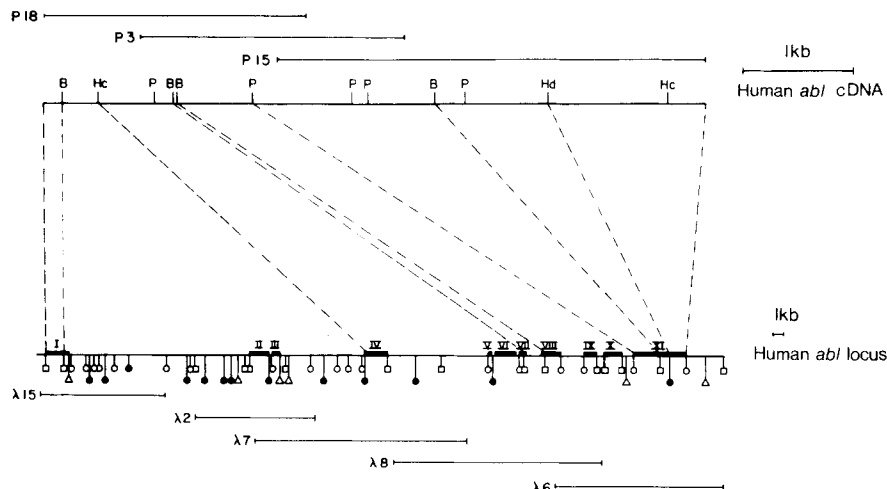
Most (90–95%) CML patients carry in their myeloid cells a unique chromosomal aberration—a shortened chromosome 22 termed the Philadelphia (Ph¹) chromosome^{2–6}. The origin of

Ph¹ was identified as a reciprocal translocation between chromosomes 22 and 9, referred to as t(9; 22) (ref. 7). The localization of the cellular oncogenes *abl* and *sis* close to or at the breakpoints on chromosomes 9 and 22, respectively^{8–10}, and the finding that the oncogenes are included in the exchanged segments of these chromosomes^{11,12} suggest that one or both are involved in development of CML. Subsequent studies failed to detect *sis* transcription in CML¹³, indicating that this oncogene does not play a role in the disease.

Mapping of the breakpoints on chromosome 9 indicated variable distances 5' (upstream) of the first known exon of *abl*. In one individual the breakpoint was 14 kilobases (kb) 5' of the oncogene, and in others it occurred further upstream and has

Fig. 1 Physical maps of normal human *abl* cDNA and locus. Restriction enzymes sites on cDNA are of *Bgl*II (B), *Hinc*II (Hc), *Pst*I (P), *Hind*III (Hd). Sites on locus are *Bam*HI (Δ), *Bgl*II ($\square$), *Hind*III ($\bullet$), *Eco*RI ($\circ$). Exons within the genomic DNA were determined by hybridization of radiolabelled cDNA fragments to fragments of the cloned genomic DNA. Exons are depicted as dark boxes delineated between restriction sites bordering the hybridizable fragments. With the exception of exon XI the size of the boxes does not reflect the length of the corresponding exons.

Methods. Analysis of cDNA was performed on the three overlapping phage clones P18, P3 and P15. A cDNA library was constructed²⁹ with poly(A) RNA isolated from the SV80 cell line²⁸. An 8- μ g sample was treated with 1 mM methylmercury for 10 min at room temperature, neutralized with 10 mM mercaptoethanol and alcohol-precipitated. The cDNA was made according to published procedures²⁹⁻³¹: the first strand was synthesized with reverse transcriptase using oligo(dT) as a primer and the second strand synthesized with a combination of the enzymes PolI, RNase H and DNA ligase. Following 'filling in' in Klenow fragment³², the cDNA was treated with *Eco*RI methylase and ligated to *Eco*RI linkers. Excess linkers were removed by selective precipitation for 20 min at room temperature in 2 M ammonium acetate with an equal volume of isopropanol, followed by agarose electrophoresis, electroelution and absorption and elution from Elutip-D-Columns. From a total of 200 ng cDNA obtained, 50 ng were used and cloned in λ gt10 vector²⁰ to yield ~200,000 recombinant plaques. Following amplification, the library was screened with a v-*abl*-specific probe³³ and nine positive plaques were detected, purified and analysed. The locus was cloned by partially digesting normal genomic DNA with *Mbo*I, selecting fragments of 10-20 kb and cloning it into the *Bam*HI site of λ L47 vector²³. Phages containing *abl* sequences were detected using the cDNA as a probe. Sites in the 2-kb gap between λ 15 and λ 2 are from ref. 14. Southern blot analysis of human DNA showed no hybridization to *abl* cDNA probe of fragments spanning the gap.



not yet been accurately mapped¹⁴. The situation on chromosome 22 is different; the breakpoints in different CMLs occurred in a restricted region of ~5.8 kb. This region was termed the breakpoint cluster region or *bcr* (ref. 15), and its consistent rearrangement in CML patients strongly implicates it in the pathogenesis of CML.

Studies of *abl* transcription provided the first strong evidence for involvement of the oncogene in CML. A novel 8-kb *abl* RNA larger than the normal species of 6 and 7 kb has been identified in the leukaemic cells of essentially all CML patients and cell lines containing Ph¹ (refs 13, 16, 17). This RNA was found both in bone marrow and peripheral blood of patients in chronic phase or blast crisis. Finally, analysis of the Ph¹-positive K562 cell line identified a new *abl* protein of relative molecular mass (M_r) 210,000, larger than the normal human species of M_r 145,000 (refs 18, 19). Unlike the normal species, the larger protein has a tyrosine kinase activity, which is a common feature of retroviral transforming proteins. By cloning and characterizing complementary DNA of the normal and CML-specific *abl* RNA, we show here the latter is a hybrid molecule containing *bcr* and *abl* sequences. Nucleotide sequencing analysis indicates that the protein translated from the fused RNA has *bcr* information at its amino terminus and that it retains most but not all of the normal *abl* protein sequence.

Cloning of normal *abl* cDNA

The direct approach to study the relationship between normal human 6- and 7-kb *abl* transcripts and the altered CML cell-specific 8-kb species is to obtain cDNA copies of the transcripts and compare them by restriction enzyme mapping. To obtain normal *abl* cDNA, RNA from the simian virus 80 (SV80) human fibroblast cell line was used to construct a cDNA library in the λ gt10 vector²⁰. The library was screened with a radiolabelled probe specific for v-*abl*, the Abelson virus homologue of the mouse *abl* gene²¹. Nine positive plaques (out of 200,000 screened) were detected, then purified and analysed. The physical map of cDNA based on three overlapping clones is shown in Fig. 1 (upper part). The cDNA is 6 kb long and ends with a stretch of poly(A) at the 3' end (B. Roe *et al.*, in preparation). It extends 520 nucleotides 5' of the beginning of v-*abl* (ref. 22) positioned eight nucleotides left of the *Hinc*II site present in clone P18. A long open reading frame begins at the 5' end of the cDNA and extends into the region homologous to v-*abl*. An

ATG identified 150 nucleotides downstream of the cDNA 5' terminus (Fig. 3; B. Roe *et al.*, in preparation) might serve as a translational initiation codon. It is not yet known whether normal human *abl* RNA contains additional sequences at its 5' end which are not included in our cDNA.

The cDNA was used as a probe to clone the *abl* locus. A partial *Mbo*I digest was prepared from white blood cells of a normal individual and, after size fractionation into 10-20-kb fragments, was cloned into the *Bam*HI site of λ L47 vector²³. A region of >50 kb was cloned in five overlapping phages and mapped with restriction enzymes (Fig. 1, bottom). The relationship between *abl* cDNA and locus was determined by hybridizing segments of the former to fragments of the latter (Fig. 1). Hence it was possible to identify 11 exons in the locus, 4 of which (I, II, IV, VI) were not detected or distinguished from other exons from a previous analysis where v-*abl* was used as a probe¹⁴. Sequences spanning the 3' two-thirds of the cDNA (delineated between a *Pst*I site and the 3' terminus) are apparently all included in a single exon, XI. The distribution of exons in the mouse *abl* locus²⁴ has common features with the situation described here for the human *abl* locus.

Cloning and sequencing of *abl* cDNA

To clone cDNA derived from the CML-specific 8-kb *abl* RNA, we used RNA extracted from the cell lines K562 and EM2. In both lines the predominant *abl* RNA is the 8-kb species (ref. 13, Fig. 4). cDNA libraries were constructed in λ gt10 vector and screened with a radiolabelled probe prepared from the 0.9-kb *Bgl*II fragment located at the 5' region of normal *abl* cDNA (Fig. 1). About 30 positive plaques of 1×10^6 screened were detected in each library; all were purified and examined by digestion with restriction enzymes.

The physical maps of two clones from each library were compared with normal *abl* cDNA (Fig. 2). Clone E6 has an identical map compared with the corresponding region on the normal cDNA, whereas clones E3, K28 & K30 share most restriction enzyme sites with the normal *abl* cDNA, but vary from the latter at the 5' region. Thus, a constellation of sites for *Ava*I, *Bgl*II and *Pvu*II enzymes present at the left end of the normal cDNA is missing from the three clones and is replaced in E3 and K28 with *Pst*I and *Hind*III sites.

To analyse further the variance between normal *abl* cDNA and K562 and EM2 cDNAs, we sequenced the 5' regions of

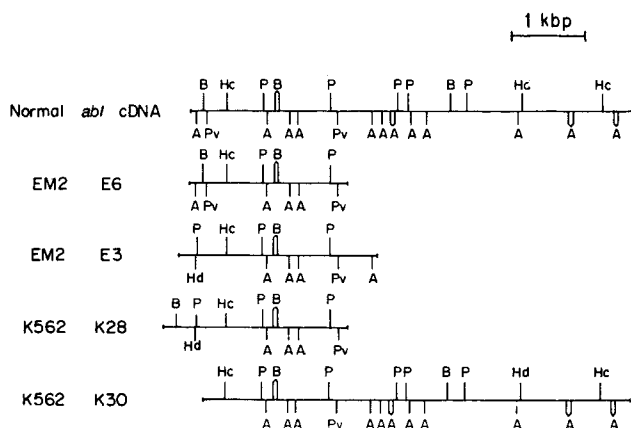


Fig. 2 Comparison between maps of normal *abl* cDNA and of *abl* cDNAs synthesized on RNA from the Ph^1 -positive K562 and EM2 cell lines. Restriction enzymes used included *Ava*I (A), *Bgl*II (B), *Pvu*II (Pv), *Hinc*II (Hc), *Pst*I (P) and *Hind*III (Hd).

these species (in clones P18, K28 and E3). The nucleotide sequences are depicted in Fig. 3 along with the sequence²⁴ of a mouse *abl* exon for comparison. The normal *abl* cDNA sequence is composed of an open reading frame extending into the region homologous to *v-abl*. K562 and EM2 have an identical sequence that diverges from the corresponding normal species in nucleotide 1. The sequence 3' to this point is shared by the three cDNAs, whereas 5' to this position the normal cDNA sequence is substituted in K562 and EM2 cDNAs with a new sequence. The inserted new DNA fragment is composed of an open reading frame that joins in phase the rest of *abl* open reading frame.

Characterization of new sequence

The finding of a new DNA linked to *abl* cDNA and substituting a portion of the coding region of the latter in CML cells suggested that the CML-specific 8-kb *abl* RNA originates from this linkage. To test this directly we subcloned into the puc13 plasmid the 0.45-kb *Eco*RI/*Pst*I fragment located at the 5' end of clone K28 (this fragment is composed entirely of non-*abl* sequences), radiolabelled it and examined its hybridization to RNAs from non-CML and CML cell lines. As a control, the blot was first hybridized to the *v-abl* probe and was found to show the normal 6- and 7-kb *abl* RNAs in all lines and the 8-kb *abl* RNA in K562 cells (Fig. 4A). The *Eco*RI/*Pst*I fragment reacted with the 8-kb species but not with normal *abl* RNAs (Fig. 4B). Two additional transcripts of ~4.5 and ~6.7 kb were apparent in all lines, in particular in SMS-SB. We conclude that cDNA clones E3, K28 and K30 originate from the 8-kb altered *abl* transcript and that the non-*abl* sequence contained in this RNA is also expressed in other human cell lines in the form of two distinct transcripts of 4.5 and 6.7 kb.

To study further the nature of the new sequence we used the *Eco*RI/*Pst*I-radiolabelled fragment to probe Southern blots of normal DNA digested with *Eco*RI, *Bam*HI and *Hind*III, and we detected single fragments of 17, 12 and 11 kb, respectively (not shown). The 17-kb *Eco*RI fragment was cloned into λ Charon 4A vector²⁵ and mapped with restriction enzymes (Fig. 5A). Examination of the map of our clone revealed that it is very similar to the physical map of the breakpoint cluster region (*bcr*) reported previously by others¹⁵. As the hallmark of this region¹⁵, located on chromosome 22, is that it is cleaved in all Ph^1 -positive CMLs (so that sequences 5' to the breakpoint remain on chromosome 22 and sequences 3' to this site translocate to chromosome 9), we tested whether our *Eco*RI/*Pst*I DNA probe would detect such breaks. DNAs from leukaemic cells of eight CML patients were cleaved with *Sst*I enzyme, electrophoresed on agarose gel, blotted onto nitrocellulose filter and hybridized to our probe. Six of eight samples showed a new fragment not detected in the normal control. Part of this analysis is shown in Fig. 5B. We conclude that the new sequence linked

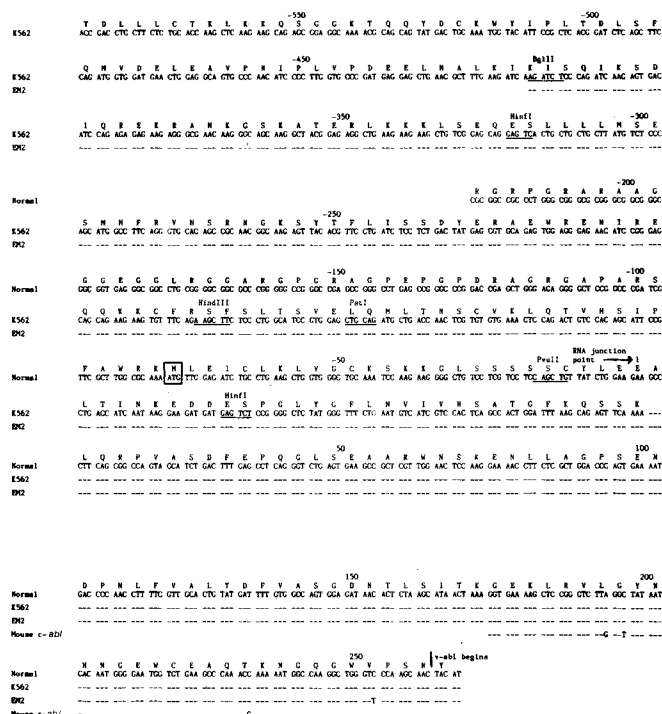


Fig. 3 Nucleotide sequence of the region of divergence between normal, K562 and EM2 *abl* cDNAs. Regions delineated between the 5' terminus and the *Hinc*II site in clones p18 (normal), E3 (EM2) and K28 ((K562) were sequenced on both strands by the Maxam and Gilbert technique³⁴. The sequence numbers are from the point of divergence between the normal *abl* cDNA and the two other species. Dashes represent the same nucleotides as in the line above; single letters above the sequence correspond to amino acids translated from the sequence; boxed ATG at position -79 on the normal cDNA could be the initiation codon for normal *abl* protein. Alternatively, initiation is in as yet uncloned sequence 5' to the beginning of clone p18. A portion of mouse *abl* sequence²⁴ is shown for comparison.

to *abl* cDNA (RNA) in K562 and EM2 lines is derived from the *bcr* and that CML-specific 8-kb RNA is a fused transcript of *abl* and *bcr*.

After cloning *abl* and *bcr* loci, we were able to map the sequences at the RNA junction point to their coding regions in the loci. This was done by isolating the *Hinf*I/*Hinc*II fragment from the K28 clone and using it as a radiolabelled probe. The fragment spans the cDNA linkage point and contains 65 and 258 base pairs of *bcr* and *abl* information, respectively (Fig. 6). The probe was annealed to a *Bgl*II + *Hind*III digest of the DNA of phage λ 2 containing exons II and III of the *abl* locus (Fig. 1). A 2-kb fragment containing exon II and a 1.1-kb fragment containing exon III reacted with the probe (Fig. 6b). This probe did not hybridize (Fig. 6a) to DNA of phage λ 15 that encloses *abl* exon I. These results show that *abl* RNA at the junction point is transcribed from exon II and that sequences of exon I are not included in the 8-kb transcript. Hybridization of the same probe to different segments of *bcr* showed a reaction (Fig. 6c) with a 1.3-kb *Bam*HI/*Hind*III DNA at 15–16.3 kb on the *bcr* map (Fig. 5a). Thus, a sequence transcribed from this area forms the RNA junction point.

Conclusions

The results described here help to explain previous observations that implicated the *abl* oncogene and *bcr* in the pathogenesis of CML. The CML-specific 8-kb RNA is a hybrid transcript of *abl* and *bcr* genes. As a result of the t(9; 22) translocation both genes are juxtapositioned on Ph^1 , conceivably in a head-to-tail configuration with the *bcr* closer to the centromere¹⁵. The 8-kb transcript presumably initiates in the *bcr* and extends into *abl* sequences by a splicing mechanism. The great variance in the distance between the chromosomal breakpoints and *abl*¹⁴

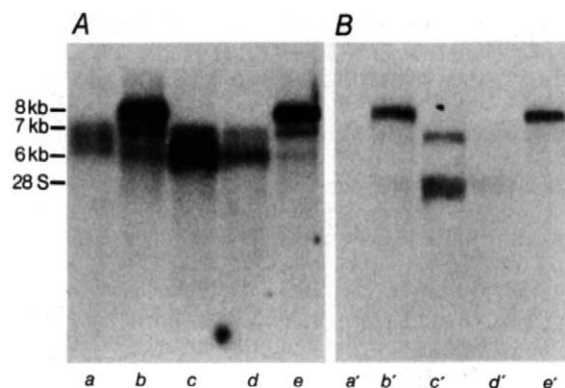


Fig. 4 Hybridization to human RNA of the sequence linked to *abl* RNA in K562 and EM2 cell lines. The blot was first hybridized to *v-abl* probe (A), then 'stripped' by washing in methyl mercury hydroxide and rehybridized (B) to the 0.45-kb *EcoRI*/*PstI* fragment from the 5' terminus of clone 28. 10- μ g aliquots of poly(A) RNA from the cell lines K562 (b, e), HL-60 (a), SMS-SB (c) and Molt 4 (d) were electrophoresed, blotted onto nitrocellulose filter and hybridized as described¹³.

(although the breakpoints are presumed always to be positioned 5' of the main body of the gene) is not reflected in the size of the hybrid transcript which seems to be identical or very similar in different CMLs¹³. The RNA linkage point in respect to *abl* sequences is either at the border between exon I and II or within exon II of *abl*. This means that although the region containing exon I is included in the Ph¹ of many CMLs¹⁵, this exon is spliced out and not included in the fused transcript of K562 and EM2 RNA and possibly in all CMLs. The *bcr* RNA at the junction point is included in an exon coded by the region at 15–16.3 kb on the *bcr* map (Fig. 5). Thus, if the RNA junction point is identical in all CMLs, this exon always has to be included in the Ph¹ and the chromosomal breakpoints must map to the right-hand side of the *Bam*HI site at 15 kb on the *bcr* map. As the breakpoints in some CMLs¹⁵ were tentatively mapped to positions left of this *Bam*HI site, the question of whether all CMLs have identical RNA junction points must await further investigation. Nevertheless, our results suggest that CMLs will have long primary *abl/bcr* fused transcripts differing in size according to the distance between *abl* and the breakpoint on chromosome 9 in a particular CML. These transcripts will be spliced into identical or similar sized mature RNAs. The splice between the exon at 15 kb on the *bcr* map and *abl* exon II could involve dozens of kilobases in some CMLs. In these cases the hybrid gene on the Ph¹ would resemble in its large size the bithorax complex of *Drosophila*²⁶.

The 8-kb RNA in CML cells will contain ~5.7 kb *abl* information. The rest of the sequence is presumably derived from the 4.5- or 6.7-kb normal *bcr* transcripts identified here. Comparison of the *bcr* sequence at the junction point (Fig. 3) with a cDNA sequence of the normal *bcr* RNA²⁷ indicates that it coincides at a position ~1,500 nucleotides from the 3' end of the latter. This suggests that the fusion partner in 8-kb RNA will be the 4.5-kb *bcr* RNA and that its 5' 3 kb are joined to the 3' 5.7 kb of *abl* to form a 8.7-kb species.

Comparison of the signal intensity of the 8-kb altered RNA and 4.5-kb normal *bcr* transcript in hybridizations using the *bcr* probe (Fig. 4) show the former to be considerably stronger (with the exception of the SMS-SB line). If, as suggested here, both transcripts initiate at the same chromosomal region, it seems that *bcr* RNA initiated on the Ph¹ (and giving rise to the 8-kb transcript) is more abundant than the 4.5- (and 6.7) kb species initiated on normal chromosome 22. Therefore, it is possible that an additional consequence of the t(9;22) aberration is the enhanced transcription of the *bcr* positioned on Ph¹.

Fusion of the reading frames of both genes in phase will result in the translation of the 8-kb RNA into a hybrid protein. The amino terminus of the normal *abl* protein (26 amino acids if initiation of the normal protein is at the ATG in position -79

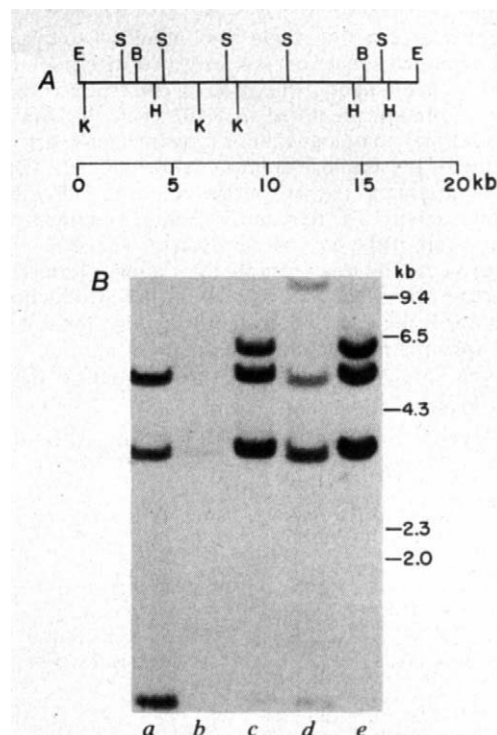


Fig. 5 Sequence linked to *abl* RNA in K562 and EM2 lines is transcribed from the *bcr*. A, Physical map of a genomic *EcoRI* fragment cloned from normal (SV80) DNA using the *EcoRI*/*PstI* probe described in the text. Sites shown are of *EcoRI* (E), *Bam*HI (B), *Kpn*I (K), *Hind*III (H), *Sst*I (S). B, Hybridization of the same probe to *Sst*I digests of genomic DNAs (30 μ g) from normal (a) and CMLs (b–e). Blots were electrophoresed and blotted by standard procedures. An 0.9-kb fragment detected at the bottom of the blot cannot be accounted for in the physical map of SV80 *bcr*, but it could result from the absence of an *Sst*I polymorphic site in SV80 *bcr*.

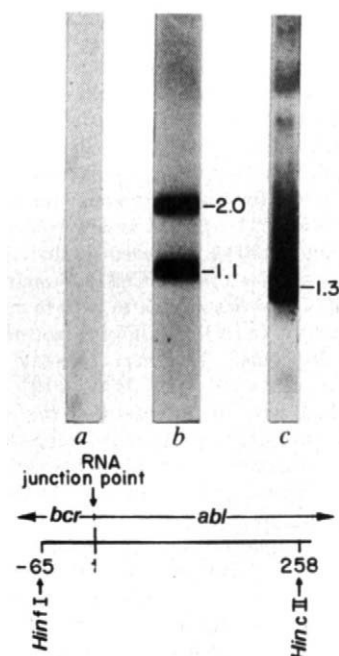


Fig. 6 Localization of sequences at the 8-kb RNA junction point on *abl* and *bcr* loci. The 0.32-kb *Hinf*I/*Hinc*II fragment from the 5' region of clone K28 (shown at the bottom) was used as a probe in hybridization with a *Bgl*II/*Hind*III digest of the λ 15 *abl* (lane a) and λ 2 *abl* clones (lane b) shown in Fig. 1. The same probe was hybridized to a *Hind*III + *Bam*HI digest (lane c) of the genomic *bcr* clone shown in Fig. 5.

in Fig. 3, or a larger polypeptide if initiation is 5' to this position in as yet uncloned sequence) is substituted with a *bcr* encoded polypeptide. Presumably, this protein corresponds to the M_r 210,000 *abl* protein identified in K562 cells^{18,19}. The protein, unlike its normal homologue, has a tyrosine kinase activity. The substitution of the amino terminus could change the conformation of the enzyme to trigger (perhaps constitutively) the phosphorylating activity. The new amino terminus could also change the localization of the enzyme in the cell.

CML provides the first example for a human cancer where a chromosomal translocation results in the production of a modified oncogene-encoded protein which is probably directly involved with the malignant process.

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Note added in proof: Sequence at the RNA junction point is derived from the 3' end of a 78-bp *bcr* exon and the 5' end of the 174-bp *abl* exon II. Therefore, the normal splice signals of the two genes are used to form the hybrid transcript.

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LETTERS TO NATURE

EXOSAT observations of a 2,000-s intensity dip in Seyfert galaxy NGC4151

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Variations in the flux from active galactic nuclei (AGN) allow the testing of models of their emission mechanisms. Various suggestions for the energy generation in AGNs require the existence of a compact object, with X rays generated deep in that object's potential well and, therefore, X-ray luminosity related directly to the object responsible for their enormous luminosities. During a 5-h X-ray observation of NGC4151 we observed a significant drop in intensity lasting ~2,000 s. The event is similar to a short flux decline previously observed¹ in NGC6814. Simultaneous intensity monitoring of a quasar with coordinates close to those of NGC4151 revealed no variability in its flux, allowing instrumental causes of the event to be discounted. The short timescale of the intensity dip imposes limits of $2 \times 10^7 M_\odot \leq M \leq 3 \times 10^8 M_\odot$ on the mass of the central black hole. We suggest here that the sudden drop in intensity may have been caused by a change in the ionization state of gas clouds surrounding the black hole or, possibly, by the passage of a star across the line-of-sight in the dense nuclear region of the galaxy.

Fourteen extragalactic objects have exhibited variability on timescales $< 10^4$ s (ref. 2 and refs therein) comparable with the light travel time across the Schwarzschild radius of a galactic nucleus-sized black hole. But in some of the X-ray observations it has been difficult to discount instrumental causes for the variability. NGC4151, one of the closest Seyfert galaxies, has been intensively studied at X-ray wavelengths³⁻⁵, with a 2-10 keV luminosity of a few $\times 10^{42}$ erg s⁻¹, a variable low-energy absorption^{6,7} as well as a low-energy excess being reported⁸. Luminosity variations on timescales of days are common⁹ and there has been some evidence of more rapid changes but at low statistical significance¹⁰.

The present observations were made on 11 July 1983 with the low-energy imaging telescopes on EXOSAT (ref. 11). The detector used was a channel multiplier array (CMA) with the thin lexan filter giving a sensitivity to X rays between 0.04 and 2.0 keV. No spectral resolution was available. NGC4151 was offset from the centre of the field-of-view by ~10 arc min. The BL Lac object 1E1207.9+3945 (ref. 12) ($z=0.59$) was also observed in the field of view ~4.7 arc min from the Seyfert galaxy. Spatial filters were used to isolate the signals from NGC4151 and 1E1207.9+3945. As the filters were fixed in spacecraft coordinates throughout the observation and defined precisely the relevant areas of sky, they exclude the possibility of all but the most contrived instrumental causes of variability. The average count rate from NGC4151 was 0.022 ± 0.005 c.p.s. and that from 1E1207.9+3945 was 0.010 ± 0.005 c.p.s. The background estimates for both the Seyfert and the BL Lac object were selected from annuli of equal area and concentric to the circular area of 1 arc min radius centred on the objects chosen (the CMA FWHM response is 18 arc). Typical background count rates were a few $\times 0.001$ c.p.s. Data obtained with the other low-energy imaging telescope were contaminated with solar-flare emission and cannot be satisfactorily analysed (the reason for the difference in the response of the two telescopes to solar flares is well understood). At the time of the observation reported, the medium-energy detectors were not operating in a mode which allows the comparison of their data with that from the CMAs. NGC4151 was detected in the higher energy gas scintillation proportional counter (GSPC) detector though at such a weak level as to preclude a search for variability in its count rate.

A search was conducted for variability in the flux from NGC4151 by binning the data with bin widths from 1,000 to 9,500 s in steps of 100 s and testing the value of χ^2 against the hypothesis of a uniform counting rate. Such a series of timelines do not represent completely independent trials and the interpretation of the χ^2 results must allow for this. The timelines may be considered independent if the phase difference between the last bins in each data set is greater than one bin width¹³. There were six independent timelines and the probability of obtaining the six values of χ^2 by chance in non-ordered samples was

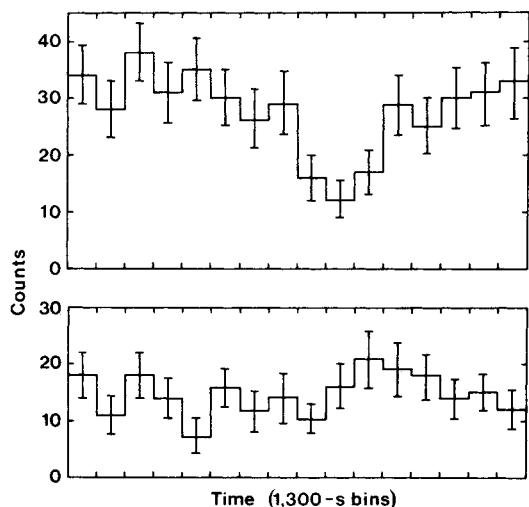


Fig. 1 The background subtracted light curves for NGC4151 (top) and 1E1207.9+3945 (bottom). Origin in each case is 7 h 3 min 6 s. Times are in UT.

$<10^{-4}$. The light curves for the observation (Fig. 1) show that the cause of the large χ^2 is a decline in the flux from NGC4151 over a $\sim 2,000$ -s period.

There are two plausible explanations for this intensity change. Either the opacity of the line-of-sight material within NGC4151 varied, perhaps because of changes in its ionization state, or an essentially opaque body passed between the X-ray source and the telescope. In either case, it is difficult to avoid the conclusion that both the X-ray source and the variable part of the intervening matter have dimensions of the order of, or less than, $2,000 c = 6 \times 10^{13}$ cm.

Halpern¹⁴ has suggested that variable absorption in QSO MR2251-178 results from changes in the ionization state of the gas surrounding the nucleus. If this model is applied to the current observations, the radial extent of the gas must be $<6 \times 10^{13}$ cm and the ionization parameter¹⁵ ξ must lie between 1 and 100 in order for substantial changes in X-ray transmission to occur. The timescales quoted by Halpern for opacity changes are similar to the timescale of the variability reported here. The low-energy count rate requires a column density $<10^{23}$ atoms cm^{-2} . Using the extreme values allowed by the data, the electron number density in the ionized region must be $\sim 10^9 \text{ cm}^{-3}$. This places the absorption cloud at 10^{15} – 10^{16} cm from the nucleus to achieve the required range of ξ .

The bolometric luminosity of NGC4151, $2.7 \times 10^{45} \text{ erg s}^{-1}$ (ref. 2) and the observed variability enable limits to be placed on the mass of the central black hole of $2 \times 10^7 M_\odot \leq M \leq 3 \times 10^8 M_\odot$, model-dependent arguments reduce the range further. The Elliot-Shapiro relationship¹⁶ is satisfied in this case indicating that beaming of the radiation is not required, but the Cavallo-Rees accretion scattering limit¹⁷ is not valid for an occulting object external to the accreting material.

Stars have photospheric radii in the range 5×10^{10} – 7×10^{13} cm (ref. 18) and are large enough to occult all, or a large fraction of, the X-ray emitting part of an accretion disk surrounding a black hole with mass in the range 3×10^4 – $2 \times 10^8 M_\odot$. The spatial density of stars close to the central objects may be of the order of $10^6 M_\odot \text{ pc}^{-3}$ (ref. 19) and therefore such occultations can occur on timescales of months or years. If this is so then the velocity of the occulting object must lie between 4×10^7 and $3 \times 10^{10} \text{ cm s}^{-1}$ to perform the occultation in the observed time. This range is appropriate for keplerian velocities within a parsec of the nucleus. We suggest that luminosity dips caused by 'star shadows' will occur, and should be searched for, regardless of whether they caused this event.

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A model of object Geminga as a degenerate white dwarf orbiting around a black hole

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Geminga (2CG195+04) is a very bright γ -ray source with the flux $F_\gamma = 2.4 \times 10^{-9} \text{ erg s}^{-1} \text{ cm}^{-2}$ for $E \geq 50 \text{ MeV}$ (refs 1-3). It has been recently identified with X-ray⁴ and optical⁵ sources. The optical object is the bluest for its magnitude⁵, indicating compactness of the emitting region. The high ratios $L/L_X \approx 10^3$, $L_X/L_{\text{opt}} \approx 200$ and the absence of radio emission are very unusual. The observed periodicity is $P \approx 1 \text{ min}$ and it changes at a rate corresponding to a doubling time of about several hundred years. I propose here a model which quantitatively explains the values of P and $\dot{P}$. It consists of a degenerate dwarf ($M_d = 0.6 M_\odot$) filling its Roche lobe and orbiting a black hole ($M_{\text{bh}} = 4.4 M_\odot$) with a period $P = 1 \text{ min}$. Gravitational radiation dissipates the angular momentum, which leads to mass transfer with a current rate $\sim 10^{-3} M_\odot \text{ yr}^{-1}$ and a period change $\dot{P}$ which agrees with the observed value. At a distance of 100 pc the flux of this radiation would be $\sim 5 \times 10^{-3} \text{ erg s}^{-1} \text{ cm}^{-2}$ (at $\nu = 1/30 \text{ Hz}$). This is about seven orders of magnitude greater than from any other known stationary source.

Let R_{12} be the distance between the dwarf and the black hole. Because the dwarf fills its Roche lobe, its radius equals⁶:

$$R_{\text{Roche}} = R_d = R_{12} \frac{2}{3^{4/3}} \left(\frac{M_d}{M} \right)^{1/3},$$

$$M = M_d + M_{\text{bh}} \quad (1)$$

We can use this and Kepler's laws to describe the orbital period of the system

$$P = \frac{2\pi R_{12}^{3/2}}{(GM)^{1/2}} = \frac{9\pi R_d^{3/2}}{(2GM_d)^{1/2}} \quad (2)$$

On the other hand, for a dwarf made of helium or heavy elements ($\mu_e = 2$) the radius R_d depends on the mass M_d through the relation⁷:

$$R_d \approx 7.6 \times 10^8 \left(\frac{M_\odot}{M_d} \right)^{1/3} \text{ cm} \quad \text{for } 0.2 \leq \frac{M_d}{M_\odot} \leq 0.75 \quad (3)$$

Therefore, we can eliminate R_d from equation (2) and write the orbital period P as a function of the mass of the dwarf only:

$$P = 36 \left(\frac{M_\odot}{M_d} \right) \text{ s} \quad (4)$$

For $P = 60 \text{ s}$ the corresponding value of M_d is $0.6 M_\odot$ and we can see that the observed period can easily be interpreted as the orbital one. To explain $\dot{P}$ let us note that the gravitational radiation leads to a loss of orbital angular momentum which in turn causes an increase of the mass transfer and an increase of the orbital period. This effect was first applied to explain some

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of the dwarf novae properties^{6,8}. The orbital angular momentum J and its change $\dot{J}$ due to the gravitational radiation are given by⁹

$$J = M_d M_{bh} \left(\frac{GR_{12}}{M} \right)^{1/2}, \quad \frac{\dot{J}}{J} = - \frac{32G^3}{5c^5 R_{12}^4} M M_d M_{bh} \quad (5)$$

Combining equations (1)–(5) we obtain the equation for M_d and its derivative $\dot{M}_d$, expressed in solar units ($m = M/M_\odot$):

$$\frac{\dot{m}_d}{m_d} = -1.3 \times 10^{-10} m_d^{11/3} m^{2/3} \frac{\left(1 - \frac{m_d}{m}\right)^2}{1 - \frac{5}{3} \frac{m_d}{m}} \quad (6)$$

The solution of equation (6) when $m_d \ll m$, together with equations (4) and (1), lead to the relations:

$$m_d = 0.6(1 + 6.3 \times 10^{-6} m^{2/3} t_d)^{-3/11} \quad (7)$$

$$\dot{M}_d = -4 \times 10^{-4} m^{2/3} (1 + 6.3 \times 10^{-6} m^{2/3} t_d)^{-14/11} M_\odot \text{ yr}^{-1} \quad (8)$$

$$P = 60(1 + 6.3 \times 10^{-6} m^{2/3} t_d)^{3/11} \text{ s} \quad (9)$$

$$R_{12} = 4 \times 10^9 (1 + 6.3 \times 10^{-6} m^{2/3} t_d)^{2/11} \text{ cm} \quad (10)$$

where t_d is the time in days with $t_d = 0$ at the moment when $P = 1$ min. The curve $P(t_d)$ given by equation (9) approximately fits the observational points for $m = 5$ (see Fig. 1). The deviations of these points from a straight line may be due to the observational errors or to some phenomena, connected, for example, with the angular momentum exchange between the orbital and intrinsic rotation of the companions. The mass $M_{bh} = 4.4 M_\odot$ is greater than the usually assumed value for the maximum mass of a neutron star of $2M_\odot$. The absence of radio emission and the very faint X-ray source also implies the absence of a neutron star in this system.

It has been suggested¹⁰ that the evolutionary path leading to the formation of a binary system consisting of a degenerate dwarf and a relativistic star (a black hole or a neutron star) may start with a close pair of main-sequence stars of mass $\sim 11 M_\odot$.

If the initial mass of the white dwarf in Geminga was $1 M_\odot$, then the filling of the Roche lobe and the onset of the mass transfer and appearance of the γ -ray source happened ~ 130 yr ago, when the parameters of the system were: $P_{\min} \approx 36$ s, $R_{12,\min} \approx 3.6 \times 10^9$ cm. So, Geminga may be a very young γ -ray source. Before the Roche lobe filling phase (for $t \leq 0$) the system had been in a very quiet state for a long time. Gravitational radiation was decreasing the orbital period and the separation, according to equation (5) but with constants M_d and M_{bh} :

$$R_{12} = R_{12,\min} \left(1 - \frac{256G^3}{5c^5 R_{12,\min}^4} M M_d M_{bh} t \right)^{1/4} \\ = 3.6 \times 10^9 (1 - 3 \times 10^{-11} m m_d m_{bh} t)^{1/4} \text{ cm} \quad (11)$$

$$P = P_{\min} \left(\frac{R_{12}}{R_{12,\min}} \right)^{3/2} \\ = 36 (1 - 3 \times 10^{-11} m m_d m_{bh} t)^{3/8} \text{ s}, \quad t \leq 0$$

If the formation of the binary system had happened $< 10^{17}$ s ago, initially it had parameters:

$$R_{12,0} \leq 1.5 \times 10^{11} (m m_d m_{bh})^{1/4} \text{ cm} \\ P_0 \leq 2.7 (m m_d m_{bh})^{3/8} \text{ h} \quad (12)$$

In the future, mass transfer in Geminga will proceed up to the moment when a planet of non-ideal matter is formed and the mass transfer will stop. It follows from equation (7) that the transfer stage will proceed for at least a Hubble time $\sim 3 \times 10^{17}$ s, after which the period will be $P \approx 2.2$ h, $R_{12} \approx 10^{11}$ cm and $M_d \approx 4.5 \times 10^{-3} M_\odot$.

The present mass transfer rate, $\dot{M} \approx 10^{-3} M_\odot \text{ yr}^{-1}$, is very high. However, if Geminga is at a distance of about 100 pc (ref. 5) then its luminosity $L_\gamma \approx L_\odot$ can be explained by a much smaller accretion rate, $\sim 10^{-9} - 10^{-11} M_\odot \text{ yr}^{-1}$, suggesting either a low efficiency of accretion or a non-stationary accumulation

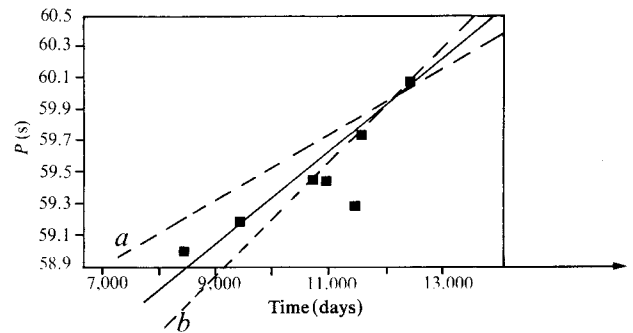


Fig. 1 The dependence of the period (in s) on time (in days). ■, Observational data¹⁹. The lines are calculated from equation (9). Solid line corresponds to $m = 5$, the dashed line a to $m = 3$, and the dashed line b to $m = 7$. All three lines meet at the point $P = 60$ s, $t = 12,120$ days.

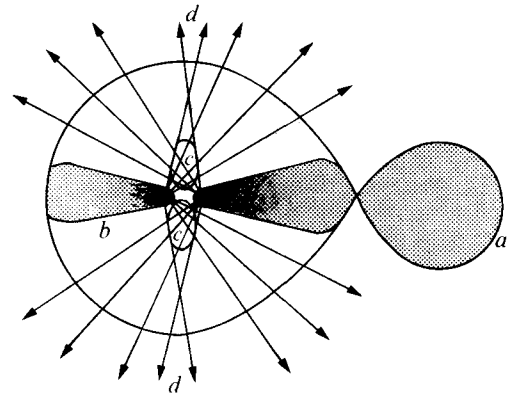


Fig. 2 The model of Geminga. a , White dwarf filling its Roche lobe; b , accretion disk around black hole; c , regions of formation of the γ -ray emission due to collisions of relativistic particles; d , the beam of relativistic particles.

of matter in the accretion disk. Both these cases have been recently treated by authors (see refs 11, 12 and those cited in ref. 13) who considered geometrically thick, low-viscosity, laminar accretion flows onto black holes ('thick accretion disks').

If the magnetic field is frozen into the matter of the disk, then the induced electric field accelerates the charged particles up to relativistic energies in the funnel of the thick disk which forms along the rotation axis¹³. The interaction of the relativistic particles may lead to the formation of π^0 mesons with the subsequent decay to γ -quanta. The qualitative picture of the model of Geminga is presented in Fig. 2. As in the Crab and Vela¹⁴ the energy losses in the form of relativistic particles may be 100–1,000 times greater than L_γ . The rotation of the thick disk must be nearly uniform so that the magnetic field will not be strongly amplified and the quasi-stationary state of the disk may be preserved for a long time.

If the X-ray flux is formed by the interaction of the relativistic flow with the surface of the dwarf and the disk, then only a small part of the energy flux reaches these surfaces. Due to the reflection effect, we may expect optical variations with the same period $P \approx 1$ min and periodical Doppler shifts of the spectral lines from the dwarf with the amplitude $\approx 4,200 \sin i \text{ km s}^{-1}$, where i is the unknown angle between the normal to the orbital plane and the line-of-sight. The optical observations may serve as a decisive test for the validity of this model.

The development of turbulence in the accretion disk may lead to a strong X-ray emission $L_X \approx L_{\text{edd}} \approx 5 \times 10^{38} \text{ erg s}^{-1}$, but the duration and the frequency of such bursts are impossible to predict within the present model.

If the other unidentified point-like γ -ray sources¹⁵ are of the same origin as Geminga, then we may expect similar L_γ/L_X and L_X/L_{opt} ratios and a periodic modulation of the γ -ray flux with $1 \text{ min} \leq P \leq 3 \text{ h}$.

The main energy losses by Geminga in this model are due to gravitational radiation. Using the standard quadrupole formula⁹ and equation (10) we may write for the gravitational wave luminosity L_G :

$$L_G = \frac{32}{5} \frac{G^4}{c^5} \frac{M_d^2 M_{bh}^2 M}{R_{12}^5} \approx 6 \times 10^{39} (1 + 1.8 \times 10^{-5} t_d)^{-10/11} \text{ erg s}^{-1} \quad (13)$$

At a distance of 100 pc, this corresponds to a value of the flux on the Earth of

$$F_G = 5 \times 10^{-3} \text{ erg cm}^{-2} \text{ s}^{-1} \quad (14)$$

This flux is about seven orders of magnitude greater than the flux from any other known binary system¹⁶.

In the case of supernovae explosions, a high deviation from spherical symmetry and high infall velocities must be assumed to allow a detectable amount of gravitational radiation. There are doubts^{17,18} over whether such high values are consistent with real supernovae events. On the other hand, our model of Geminga employs simple arguments and realistic parameters

for the binary system. Thus the search for gravitational radiation (at $\nu \sim 1/30$ Hz) from Geminga would probably not be less reasonable than the search for the pulses of gravitational radiation from supernova explosions.

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General relativistic effects on the pulse profile of fast pulsars

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The recent discovery of millisecond pulsars^{1,2} has led to several theories about the structure³⁻⁸ and radiation characteristics⁹⁻¹¹ of rapidly rotating neutron stars. The rapid rotation rate implies that such pulsars will be compact⁵ (implying large space-time curvature) and that surface emission characteristics will differ significantly from the non-rotational (Schwarzschild) case¹¹. We report here the implications of large space-time curvature and large rotation (specifically, the dragging of inertial frames introduced by rotation) on the trajectories of photons, and hence, on the pulse profile of fast pulsars. Space-time curvature leads to substantial amounts of divergence in the pulse width and reduction in the pulse intensity whereas rotation produces a tilt of the pulse cone from its original direction of emission, and deforms the cone, introducing an asymmetry in the (flattened) pulse profile leading to a time delay in the arrival of photons emitted within the pulse cone. We find that the effect of curvature dominates over that of rotation, suggesting a larger brightness temperature than inferred observationally for all pulsars whatever the rotation rate (unless the pulsar is of very low mass).

The full rotational analogue to the Schwarzschild space-time, namely the Kerr metric, does not have a corresponding appropriate interior solution amenable to a straightforward theoretical treatment. However, a rotationally-perturbed interior spherical metric of the following form^{12,13} is adequate (signature +---)

$$ds^2 = g_{\alpha\beta} dx^\alpha dx^\beta = e^{2\nu} dt^2 - e^{2\psi} (d\phi - \omega dt)^2 - e^{2\mu} d\theta^2 - e^{2\lambda} dr^2 \quad (1)$$

where ω is the angular velocity of the cumulative dragging of inertial frames and ν , ψ , μ , λ depend on the structure of the rotating neutron star. This metric is valid for strong gravitational fields and for a uniform rotation rate that has as its upper bound the critical speed for centrifugal breakup. Neutron star models which rotate at the secular instability limit (assuming the star to be homogeneous) and which are relevant to fast pulsars, are within this bound⁶.

Rotating neutron-star structure parameters using the metric (1) (see refs 5, 6) can be used in an external metric (which

match at the surface) for calculating the photon trajectories. Space-time curvature will bend a photon trajectory, and rotation will drag it away from its original direction towards the direction of rotation. The net angle of deflection in the trajectory will be given by

$$-\phi_0(\delta) = \int_{r_e}^D \frac{d\phi/d\Gamma}{dr/d\Gamma} dr \quad (2)$$

where Γ is an affine parameter and r_e and D are respectively the radial locations of the emission and reception of the photon. The deflection angle is a function of δ , the azimuthal angle at which the photon is emitted with respect to the radius vector of the source through the origin of coordinates as seen in the local rest frame of the star ($\delta=0$ corresponds to a radially outgoing photon and $\delta=3\pi/2$ to a tangentially-forward photon). We have neglected the polar bending ($d\theta/d\Gamma=0$) which will be small because we use a perturbed spherical geometry. Therefore, the polar angle of emission, θ_s , made by the line-of-sight through the centre of the star with respect to the rotation axis can be taken to be identical to the angle of inclination seen by a distant observer.

The lagrangian for the metric (1) is

$$L = \frac{1}{2} \{ e^{2\nu} \dot{t}^2 - e^{2\lambda} \dot{r}^2 - e^{2\mu} \dot{\theta}^2 - e^{2\psi} (\dot{\phi} - \omega \dot{t})^2 \} \quad (3)$$

where the overdot indicates derivative with respect to Γ . The equations of motion corresponding to equation (3) provide $\dot{\phi}$ and $\dot{t}$ whereas $\dot{r}$ is obtained from the null condition. Evaluated thus, equation (2) becomes

$$-\phi_0(\delta) = \int_{r_e}^D f(r, q_e) dr \quad (4)$$

$$f(r, q_e) = \frac{\omega(1 + \omega q_e) - q_e e^{2\nu-2\psi}}{e^{\nu-\lambda} \{ (1 + \omega q_e)^2 - q_e^2 e^{2\nu-2\psi} \}^{1/2}} \quad (5)$$

Here q_e is the impact parameter of the photon at the emission location, and has the form¹¹

$$q_e = \frac{e^{\psi-\nu} (v_s + \sin \delta)}{1 + e^{\psi-\nu} (\omega v_s + \Omega \sin \delta)} \Big|_{r=r_e} \quad (6)$$

where Ω is the angular velocity of the star as seen by a distant observer and

$$v_s = e^{\psi-\nu} (\Omega - \omega) \quad (7)$$

Because of bending (see Fig. 1), the space-time curvature will cause a divergence in the pulse width. As a measure of this

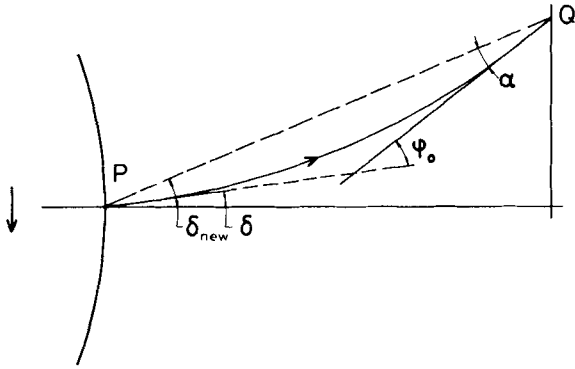


Fig. 1 Schematic illustration of the trajectory of photon starting from P at (or near) the pulsar surface in terms of the azimuthal angle of emission (δ) and bending ($\phi_0 \equiv \delta_{\text{new}} - \delta$). For an observer at infinity (denoted by Q), the angle $\alpha \rightarrow 0$. The arrow implies the direction of rotation of the neutron star.

divergence effect, we introduce a parameter Δ defined as

$$\Delta = (d\delta_{\text{new}}/d\delta)_{r=r_e} = (1 + d\phi_0/d\delta)_{r=r_e} \quad (8)$$

where

$$\delta_{\text{new}} = \delta + \phi_0(\delta) \quad (9)$$

As a consequence of rotation, the impact parameter is asymmetric in $\pm\delta$ and

$$\Delta(\delta, r) \neq \Delta(-\delta, r)$$

implying an asymmetry in the pulse profile (assuming the initial profile to be symmetric). This is unlike the Schwarzschild case, where although the space-time curvature will produce a pulse divergence effect of similar extent, there will be symmetry in $\pm\delta$.

The pulse divergence described above will imply a reduction in the pulse intensity, which can be understood in terms of conservation of energy flux. We denote the intensity deamplification factor by ε :

$$\varepsilon = \frac{I(\delta_{\text{new}})}{I(\delta)} = \frac{1}{\Delta} \frac{\sin \delta}{\sin \delta_{\text{new}}} \quad (10)$$

The factor ε must be corrected for redshift effects as follows, using Liouville's theorem¹⁴. If I_ν is the observed specific intensity and I_{ν_0} the emitted (monochromatic) specific intensity (at a given δ), then

$$I_\nu = I_{\nu_0} (\nu/\nu_0)^3 = I_{\nu_0} (1+z)^{-3} \quad (11)$$

where $(1+z)$ is the redshift factor¹¹:

$$1+z = \frac{1 + \Omega q_e}{e^{\nu(1-v_s^2)^{1/2}}} \quad (12)$$

The redshift correction to the intensity through a factor $\{1+z(\delta=0)\}^{-3}$ is inherent to the pulse. Apart from this, the intensity will become enhanced for forward emitted photons ($\delta < 0$) and reduced for backward emitted photons ($\delta > 0$). We define a net intensity deamplification factor ε' that corrects ε for Doppler boosting (for $\delta < 0$) and diminution (for $\delta > 0$) as

$$\varepsilon' = \varepsilon \left\{ \frac{1+z(0)}{1+z(\delta)} \right\}^3 \quad (13)$$

in accordance with Liouville's theorem.

To illustrate the effects of space-time curvature and rotation on the pulse profile, we consider PSR1937+214, assuming its mass to be $1.4 M_\odot$ ($M_\odot$ = solar mass). For this pulsar, $\Omega = 4.0334 \times 10^3 \text{ rad s}^{-1}$; the relevant structure parameters for the

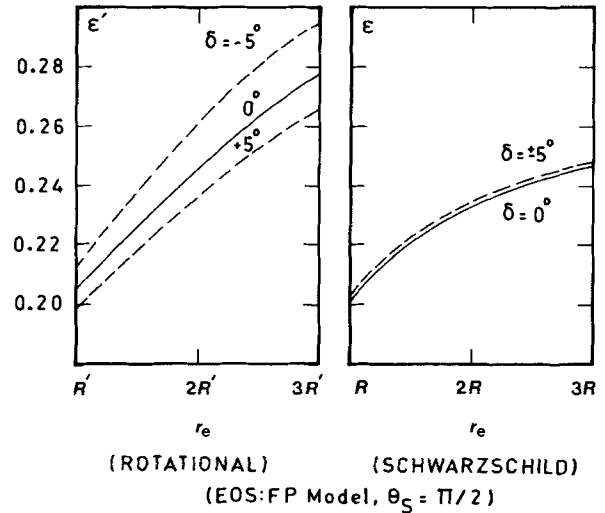


Fig. 2 Net deamplification factor (ε') versus φ radial emission location (r_e). EOS: FP model, $\theta_s = \pi/2$.

calculation have been taken from our earlier work^{5,6} (corresponding to the Friedman-Pandharipande equation of state of neutron-star matter). As this pulsar is close to the point of secular rotational instability^{5,6} the results will refer to maximal effects of rotation.

There is no general consensus on the mechanism of pulse emission. Here, we have selected one of the more popular models¹⁵, namely, that in which the emission (at a particular frequency) emanates from a point near the surface of the pulsar in the form of a narrow cone (of angle $\leq 10^\circ$) which rotates with the pulsar, sweeping the pattern past the observer. For the purposes of calculation, we take the initial pulse to be a gaussian curve with $-5^\circ \leq \delta \leq +5^\circ$.

The results of our calculations for the rotational (for $\theta_s = 0.3\pi$, 0.5π) and Schwarzschild ($\theta_s = 0.5\pi$ here) cases are summarized in Tables 1 and 2. The initial pulse becomes wider by a factor ~ 2 . There is an asymmetry in the pulse profile brought about by rotation which manifests itself in the pulse cone getting compressed for backward emission and stretched for forward emission. This asymmetry, characterized by $|\Delta(+\delta) - \Delta(-\delta)|$, is

Table 1 Azimuthal bending, divergence index (Δ) and deamplification factor (ε') for the rotational and Schwarzschild cases with emission taken at the pulsar surface considered for PSR1937+214

θ_s	δ (deg)	δ_{new} (deg)		Δ		ε'	
		+	-	+	-	+	-
Rotation case							
0.3π	0	0	0	2.512	2.512	0.158	0.158
	1	2.509	-2.514	2.507	2.517	0.158	0.159
	2	5.014	-5.034	2.503	2.522	0.157	0.160
	3	7.515	-7.558	2.498	2.527	0.157	0.161
	4	10.010	-10.087	2.494	2.532	0.156	0.162
	5	12.502	-12.621	2.489	2.537	0.156	0.164
0.5π	0	0	0	2.213	2.213	0.204	0.204
	1	2.210	-2.215	2.208	2.217	0.203	0.206
	2	4.416	-4.435	2.203	2.222	0.202	0.207
	3	6.617	-6.659	2.199	2.227	0.201	0.209
	4	8.813	-8.889	2.194	2.232	0.200	0.210
	5	11.005	-11.124	2.190	2.237	0.199	0.212
Schwarzschild case							
0.5π	0	0	0	2.226	2.226	0.202	0.202
	1	2.226	-2.226	2.226	2.226	0.202	0.202
	2	4.453	-4.453	2.226	2.226	0.202	0.202
	3	6.679	-6.679	2.227	2.227	0.202	0.202
	4	8.906	-8.906	2.227	2.227	0.202	0.202
	5	11.133	-11.133	2.227	2.227	0.203	0.203

Table 2 Rotation-induced pulse tilt angle and time delay for two values of θ_s and $r_e = R', 2R', 3R'$ considered for PSR1937+214

r_e	θ_s (deg)	Tilt angle (deg)	Time delay for δ (μ s)				
			$\pm 1^\circ$	$\pm 2^\circ$	$\pm 3^\circ$	$\pm 4^\circ$	$\pm 5^\circ$
R'	54	10.262	0.2	0.5	0.7	1.0	1.2
	90	10.253	0.3	0.6	1.0	1.3	1.5
$2R'$	54	20.199	0.8	1.6	2.4	3.1	3.9
	90	20.322	1.0	1.9	2.8	3.9	4.7
$3R'$	54	28.823	1.5	3.1	4.6	6.1	7.7
	90	29.290	1.8	3.6	5.4	7.2	9.1

proportional to δ . This is also evident in ϵ' . Figure 2 illustrates (for $\delta = 0^\circ, \pm 5^\circ$) the variation $\epsilon'(r_e)$, r_e being taken in units of $R'(R)$ which is the radius of the rotating (non-rotating) neutron star.

One interesting result of our calculations is the 'sweeping' of the entire pulse cone in the direction of rotation of the pulsar, thus imparting a tilt to the cone. The tilt can be understood in terms of the net bending of the radially outward $\delta = 0$ photon, whose values for various θ_s and r_e are given in Table 2.

In Schwarzschild space-time, photons emitted at $\pm\delta$ would have the same time of flight to the distant observer. But with the neutron star rotating rapidly, this symmetry will not be maintained. The backward emitted photons would have larger time of flight than the forward emitted photons. The difference in the arrival times of $\pm\delta$ photons, which we call time delay, can be evaluated from the equations of motion of photons in the external space-time and their redshifts. These values are listed in Table 2. If the time delay between photons with maximum δ and minimum δ equals the pulse duration, the complete pulse will be detected by the observer. If, however, this time delay exceeds the pulse duration, then, photons with δ larger than a critical δ will miss the detector. As a result, the pulse will have a gradual buildup but a steeper falloff. This will be a contributory factor to the asymmetry in the final pulse profile (apart from the effects resulting from inertial frame drag and interstellar/planetary scintillation, if any), and, in principle, will constrain the duty cycle of the pulsar.

The effects of curvature and rotation on the pulse profile presented here are purely general relativistic effects, and will be additional to any (frequency dependent) effect on the propagation of electromagnetic waves through anisotropic/inhomogeneous magnetized plasma. Observationally, most pulsars show narrow profiles. Our calculations, therefore, can be taken to imply that at the emission location, the pulse starts out in the shape of a narrow spike. As the brightness temperature of the source is directly proportional to the intensity of radiation, the important conclusion that follows is that the brightness temperatures of pulsars in general (since the curvature effects are dominant over the rotational effects) are larger by an order of magnitude (in the emitter's frame of reference) than are presently presumed.

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Possible precipitation of ice at low latitudes of Mars during periods of high obliquity

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Most of the old cratered highlands of Mars are dissected by branching river valleys that appear to have been cut by running water^{1,2} yet liquid water is unstable everywhere on the martian surface. In the equatorial region, where most of the valleys are observed, even ice is unstable^{3,4}. It has been suggested, therefore, that Mars had an early denser atmosphere with sufficient greenhouse warming to allow the existence of liquid water⁵. Here, we suggest instead that during periods of very high obliquities, ice could accumulate at low latitudes as a result of sustained sublimation of ice from the poles and transport of the water vapour equatorwards. At low latitudes, the water vapour would saturate the atmosphere and condense onto the surface where it would accumulate until lower obliquities prevailed. The mechanism is efficient only at the very high obliquities that occurred before formation of Tharsis very early in the planet's history, but limited equatorial ice accumulation could also have occurred at the highest obliquities during the rest of the planet's history. Partial melting of the ice could have provided runoff to form the channels or replenish the groundwater system.

That some mechanism must exist for replenishment of the equatorial inventory of water has been suspected on several grounds: (1) if the valleys formed in a manner analogous to terrestrial river valleys, by slow erosion of running water, then the volumes of water that would have flowed through them would have been so large that some recycling of water is implied; (2) although groundwater seepage has probably had a major role in formation of the valleys⁶, several morphological characteristics suggest that precipitation was also involved, so that transfer of water from the atmosphere to the surface occurred²; (3) the unique characteristics of several martian landforms, particularly those formed by impact, have been attributed (although not uniquely) to the presence of ground ice in regions where it is unstable.

We have calculated the potential loss of water from the poles on the assumption that sublimation of the seasonal CO₂ cap during summer exposes a residual water-ice cap, as is currently the case for the North Pole^{7,8} and may occasionally be the case for the South Pole⁹. The procedure followed here is similar to that used by Toon *et al.*¹⁰. Temperatures at the surface and down to depths of 7 m are calculated for $\pm 85^\circ$ lat. taking into account incident solar radiation, long-wave emission from the surface and atmosphere, conduction into the ground, sublimation and condensation of carbon dioxide and evaporation of water. Atmospheric pressures greater than the present value may occur at high obliquities as a result of desorption of CO₂ from the high-latitude regolith¹¹. We assumed that the pressure was 40 mbar at 45° obliquity, 25 mbar at 35° obliquity and 10 mbar at the current 25° obliquity¹². The main difference between our model and that of Toon *et al.* is that we included growth and decay of the CO₂ cap as an integral part of our model rather than assuming that the CO₂ cap disappeared at a specific time. Our results are conservative in that they give estimates of the

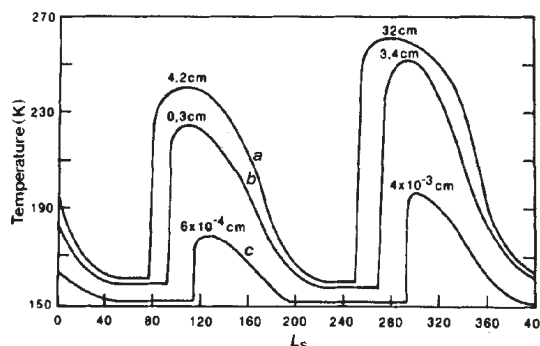


Fig. 1 Seasonal polar cap temperatures calculated for obliquities of 45° (a), 35° (b) and 25° (c). The adjacent numbers refer to the thickness of ice sublimed from the cap (in cm) during the entire summer. For clarity, only temperatures for the warmer pole are shown at each season. Calculations assume the maximum obliquity (0.14) and have periapsis at southern solstice, and thus yield the maximum differences between the two poles; other choices would yield temperatures intermediate between these extremes.

amount of water sublimed from the summer cap that are up to a factor of 10 lower than the estimates of Toon *et al.*¹⁰.

Typical results are shown in Fig. 1 for the three values of the obliquity. Although the results are sensitive to factors such as the albedo of CO₂ and water frost, the wind speed and the assumed thermal conductivity, the results, in general, demonstrate that at 45° obliquity a few centimetres to a few tens of centimetres will be evaporated from the poles during each summer. At an obliquity of 35° a few tenths of a centimetre to a few centimetres will be lost, while in present conditions only 10⁻²–10⁻⁴ cm are lost.

The results shown in Fig. 1 were calculated assuming sublimation of water into a dry atmosphere. The actual amount of water sublimed and its eventual fate will depend on the rate of atmospheric transport away from the pole. Figure 2 shows the mid-northern-summer latitudinal distribution of water vapour during 1976. Polar abundances of about 100 precipitable (pr) μ m were observed, consistent with an almost saturated atmosphere^{8,13}. Non-polar atmospheric water indicates transport of water away from the pole and/or exchange of water between the regolith and atmosphere^{14,15}, although transport from the poles ultimately dominates the process⁵. For equatorial and mid-latitude mixing timescales indicated by dynamic models^{16,17}, an amount of the order of 5×10^{14} g H₂O can be transported southwards each year, equivalent to removing $\sim 4 \times 10^{-2}$ g cm⁻² from the cap^{15,18}. Given the uncertainties in the models, this value is consistent with the estimated amounts of water that can be sublimed.

At high obliquity, a similar atmospheric circulation pattern should exist, with the exceptions of a poleward shift in the Hadley cell to the subsolar point, and more vigorous cross-equatorial transport (ref. 19 and R. M. Haberle, personal communication). Therefore, if we assume a similar mid-summer distribution of water with latitude due to transport from the polar region, we can use Fig. 2 to show the latitudinal variation at midsummer, reading now from the right-hand scale. The estimate of 5×10^3 pr μ m over the pole is conservative, based on saturation of the atmosphere over the pole at temperatures that are relatively low for 45° obliquity but higher than that of the present summer cap. By scaling the amount transported to the total abundance, the amount of water transported away from the cap is equivalent to removal of ~ 2 g cm⁻² from the pole, again consistent with the amounts estimated to have sublimed. From the right-hand scale of Fig. 2, there can be $>1,000$ pr μ m of atmospheric water at mid- and low latitudes. For less conservative assumptions, the amount of water ice sublimed can be as great as 20 cm and the water content of the atmosphere at the equator can be as high as 10,000 pr μ m.

At the equator a dust-free atmosphere is capable of holding only as much as 100–200 pr μ m of water vapour without saturat-

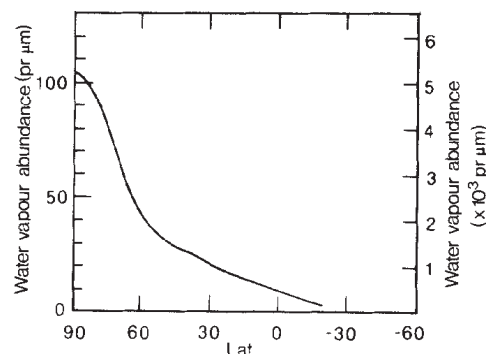


Fig. 2 Latitudinal distribution of water vapour at mid-summer in the north as observed from the Viking space craft (left-hand ordinate). The right-hand ordinate is appropriate for high values for the obliquity, when $\geq 5 \times 10^3$ pr μ m water may be present over the cap.

ing^{20,21}. Thus, the excess above this value will condense and form clouds. Persistent dust storms are, however, likely at high obliquities⁵. The presence of dust causes atmospheric temperatures to rise. As a result, the atmosphere could hold 5,000–10,000 pr μ m during the day without saturating, and up to 300 pr μ m at night²¹. Thus, even during dust storms water would condense out at night if abundances were higher than ~ 300 pr μ m. If the dust acts as condensation nuclei for the water, then the dust and ice will be removed rapidly to the surface, possibly within several days. Two uncertainties are the effect of the ice clouds on the holding capacity of the atmosphere and the effect of surface ice. Temperatures that occur in the presence of clouds are not well determined; low-altitude clouds will cool the surface dramatically^{22,23} while high-altitude clouds could increase surface temperatures¹⁰. The presence of high-albedo ice on the surface would significantly lower surface and atmospheric temperatures, thereby providing a positive feedback and enhancing the tendency for atmospheric water to condense.

If as much as 20 cm of ice are removed in 1 yr from the summer cap and deposited uniformly between $\pm 45^\circ$ latitude, the resulting ice thickness is 0.4 cm. It is uncertain, however, where the ice is deposited. The atmospheric circulation pattern and the relative timescales for condensation and settling will determine whether the ice is deposited near the edge of the summer or winter cap or more uniformly over the globe. During the following winter, the non-polar water vapour abundance at saturation will not change significantly, while the winter polar cap will have essentially no water vapour above it. The latitudinal gradient of water during winter is thus towards the pole, but is a factor of 5–50 smaller in magnitude than during summer, so that the rate of transport of water from low latitudes to the winter pole is probably low. Also, at the time when water is seeking to move back to the winter pole, additional water is being supplied from the other summer pole, further inhibiting evaporation. Ice will thus tend to accumulate in non-polar regions.

The thickness of ice that could accumulate at low latitudes and the fraction of the obliquity cycle during which it would be stable depends on the amount of ice available at the poles and the range of the obliquity variations. Before the formation of Tharsis the mean obliquity was as high as 35° and the peak value as high as 45° (ref. 24). During the highest obliquities of this epoch, 2 km of ice could be removed from the poles in $<10^4$ yr to form ice deposits tens of metres thick in the non-polar latitudes, if that much polar ice is available. Ice may have returned to the poles only at minimum obliquities. During most of Mars' history, however, obliquities ranged from 15 to 35° (refs 24, 25). Net accumulation of ice in non-polar areas is then likely only at peaks in the obliquity cycle, and removal of as much as 2 km of ice from the poles is unlikely. The polar layered deposits could, however, be extensively reworked, as is suggested by the scarcity of superimposed impact craters^{5,10}.

Meltwater from the ice deposits may have contributed to the erosion of the branched valley networks. For melting temperatures to be reached at the surface by greenhouse warming, atmospheric pressures near 1 bar are required²⁶. However, melting of snow or ice does not necessarily require surface temperatures to be above melting. The low thermal conductivity of snow and its partial transparency to sunlight can result in subsurface melting when surface temperatures are well below freezing²⁷. Thus, melting can occur in substantially thinner atmospheres than are required by the atmospheric greenhouse effect alone. Another appealing reason to invoke a mechanism that requires high obliquities to explain the valley networks is that most of the networks formed very early in Mars' history^{2,28}. At that time, the Tharsis bulge may not have reached its present size. As Tharsis grew, the maximum obliquities reached decreased, and as a result so did the efficacy of the equatorward transfer of water. Young valley networks are, therefore, rare.

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Onset of the south-west monsoon and sea-surface temperature anomalies in the Arabian Sea

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In 1979, the year of the Global Weather Experiment, the atmosphere and ocean of the monsoon region were particularly well observed. For this reason, the onset period of the monsoon, 11–19 June 1979, was selected for international comparison of numerical prediction models¹. Previous studies^{2–4} have demonstrated that successful predictions for this period are difficult to achieve. I report here two examples of numerical predictions that highlight the role of sea-surface temperatures, namely a control forecast using climatological sea-surface temperatures and an anomaly forecast using more realistic (and warmer) surface temperatures specified for the eastern Arabian Sea. It is shown that the use of the more accurate sea-surface temperatures enables a better prediction of the development of a monsoon depression to be obtained.

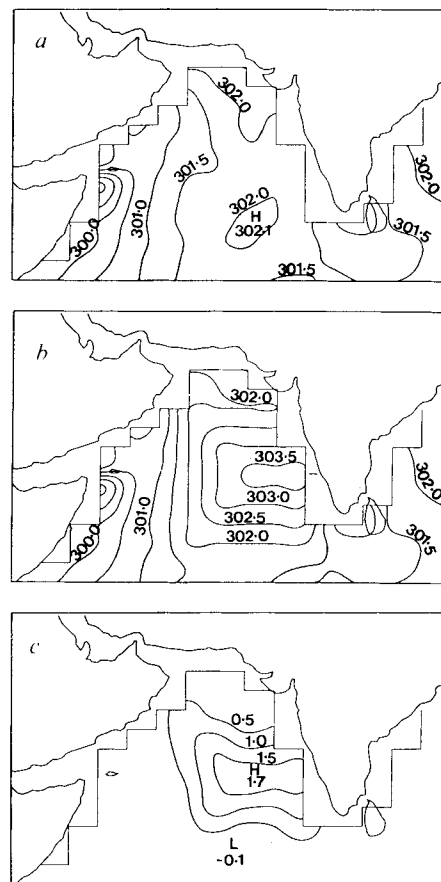


Fig. 1 *a*, Sea-surface temperature for control forecast; *b*, sea-surface temperature for anomaly forecast; *c*, sea-surface temperature anomaly (*b* - *a*). Contour interval, 0.5 K.

For these experiments, the 11-layer global atmospheric general circulation model^{5,6} of the UK Meteorological Office was used with a horizontal resolution of 2° lat. and 3° long. The initial conditions for both forecasts were taken from the (level 3B) analysis for 12.00 GMT on 11 June 1979 made by the European Centre for Medium Range Weather Forecasts (ECMWF) using data which had been collected for the Global Weather Experiment. The sea-surface temperatures were fixed throughout the 8 days of each forecast. The values in Fig. 1*a* were derived from a climatological average for the decade 1951–60; those for the eastern Arabian Sea in Fig. 1*b* were based on an analysis by Seetaramayya and Master⁷ for 8–15 June 1979. The difference chart (Fig. 1*c*) shows a maximum warm anomaly of 1.7 K in this region.

The onset of the monsoon is characterized by an increase in wind speeds at low levels over the Arabian Sea and the commencement of the rains along the west coast of India. Figure 2*a, b* illustrates the changes in the wind field at the 850-mbar pressure level during the onset period. On 11 June the south-westerly jet had a maximum speed of 14 m s⁻¹ a few degrees to the east of the Somali peninsula, and the main branch of the monsoon flow was still to the south of India (Fig. 2*a*). During the next 8 days the jet strengthened and extended eastwards to the southern tip of India. A depression formed on the northern flank of the jet and moved first north then west, allowing the monsoon westerlies to advance northwards to cover much of the Indian peninsula and bring the rains to the west coast. By 19 June the maximum speed of the jet was 29 m s⁻¹ and the cyclonic vortex of the depression was near the Arabian coast (Fig. 2*b*).

In the control forecast, the jet strengthened and extended eastwards more slowly than actually occurred, to give a maximum speed of only 24 m s⁻¹ by 19 June (Fig. 2*c*). A weak vortex developed in the correct location, but the model failed to intensify it and kept it stationary. The anomaly run produced

a much better prediction of the intensification of the vortex and moved it northwestwards. By day 8, 19 June, the centre was at approximately the right latitude but $\sim 5^\circ$ too far east (Fig. 2d). The jet strengthened more than in the control integration, giving a maximum speed of 29 m s^{-1} .

The direct effect of increasing the sea temperatures is to

increase the transfer of heat and moisture from the ocean into the atmosphere. Initially the increases are quite small, but after 8 days the differences between the anomaly and control runs (Fig. 3a, b) show maxima of 54 W m^{-2} for the sensible heat flux and 16 mm per day (460 W m^{-2}) for the rate of evaporation. The higher values in the anomaly forecast are consistent with

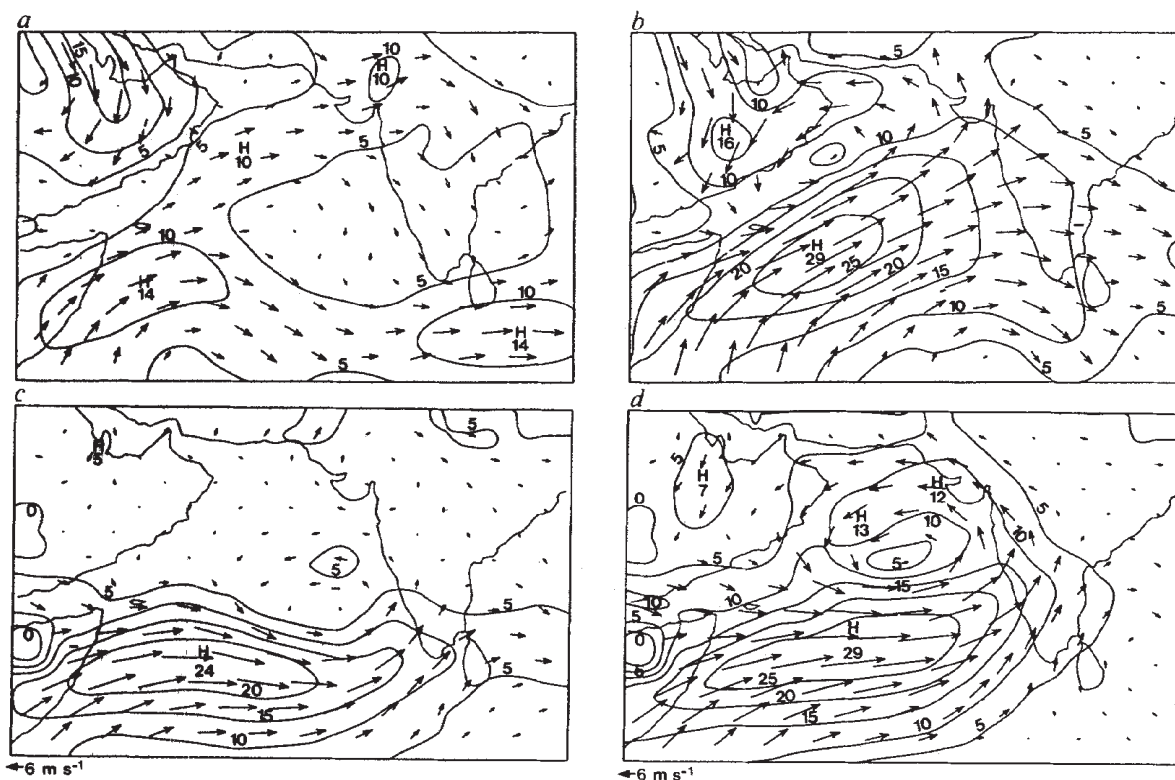


Fig. 2 Wind vectors and isotachs at 850-mbar pressure level (m s^{-1}). *a*, ECMWF analysis for 12.00 GMT 11 June 1979; *b*, ECMWF analysis for 12.00 GMT 19 June 1979; *c*, control forecast for 12.00 GMT 19 June 1979; *d*, anomaly forecast for 12.00 GMT 19 June 1979.

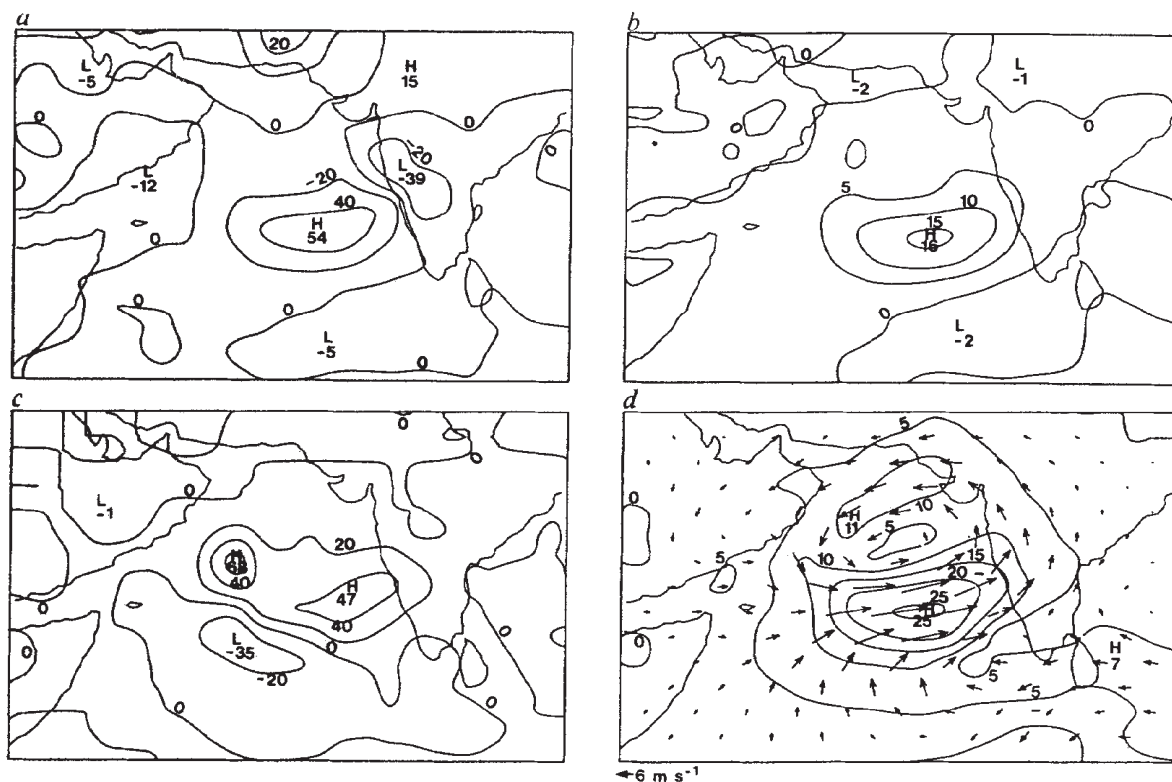


Fig. 3 Anomaly forecast minus control forecast for 12.00 GMT 19 June 1979. *a*, Upward sensible heat flux from surface (W m^{-2}); *b*, rate of evaporation from surface (mm per day); *c*, rate of precipitation (mm per day); *d*, vector wind at 850-mbar pressure level (m s^{-1}).

estimates of the fluxes for the onset period made from ship observations⁸. The changes in surface fluxes induce a substantial increase in precipitation. Figure 3c shows a maximum local increase of >65 mm per day after 8 days. This increase in precipitation is sustained partly by the increase in evaporation but mainly by increased moisture convergence. The additional latent heat released into the atmosphere is equivalent to a vertically averaged heating rate of around 16 K per day. This heating is compensated by adiabatic cooling associated with increased ascent. The response of the low-level wind field to these changes is shown in Fig. 3d. The main effect is confined to the Arabian Sea, with an asymmetrical cyclone centred to the north of the heating zone; the strongest flow is the westerly directly over this zone, and weak easterlies are induced to the south-east.

Figure 3d is qualitatively similar to Gill's^{9,10} solutions for the response of a simple linear model to prescribed heating north of the Equator. The vertical structure of the response to the increased sea temperatures is also similar to that imposed in Gill's model. The vertical velocity shows a single peak in mid-troposphere and the flow in the upper troposphere is anticyclonic above the low-level cyclone. This correspondence suggests that,

in the general circulation model, the latent heat release is the critical factor in promoting the changes in the flow, although the mechanism is not simple. A positive feedback cycle is operating, whereby the increase in latent heat release leads to increased ascent and more moisture convergence at low levels. The ascent causes increased cyclonic flow at low levels, as in the simple model, and the stronger wind increase the surface fluxes of heat and moisture. These effects collectively lead to more precipitation, completing the cycle.

A much better prediction of the development of a monsoon depression over the Arabian Sea was obtained by specifying more accurate sea-surface temperatures in the region where the depression was formed. The strengthening of the low-level jet and the northward movement of the monsoon rains along the west coast of India were also greatly improved in the forecast with the warm anomaly. These features make this prediction the best yet achieved for the onset of the monsoon in 1979. The results have clearly indicated that correct sea-surface temperatures can be essential for accurate numerical weather prediction for the tropics.

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1,200-Myr impact-melting age and trace-element chemical features of the Yamato-790964 chondrite

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Age determinations of impact-produced materials in brecciated meteorites¹ are particularly important for understanding the evolutionary history of meteorites. The age characteristics of these meteorites have been examined mostly by the K-Ar method or, in a few cases², by the Rb-Sr method. However, age data are still rare and, except for a few cases³, the large-ion lithophile (LIL) trace-element chemical features have not been studied in detail. In this respect, the Yamato-79 shocked meteorites are the best specimens for investigation. We report here results of Rb-Sr systematics and trace-element analyses for an almost wholly impact-melted LL-chondrite, Y-790964, one of the Yamato-79 unusual chondrites. The analyses yield an internal isochron age of $1,200 \pm 50$ (2 σ) Myr. The isotopic and trace-element data obtained represent the first clear evidence of the youngest impact-melting and LIL-element fractionations for a complete LL-chondrite, and suggest that the 1,200-Myr-age is a non-trivial marker in the evolution of meteorites.

The meteorite, originally weighing 3.3 kg, has a porous and microcrystalline texture with euhedral, chemically zoned pyroxene and olivine and minor relic chondrules^{4,5}, and has a similar major-element composition to that of normal LL-chondrites⁶. Two whole rocks from the different portions (A and B) and eight mineral separates from portion A of the meteorite were examined for Rb-Sr systematics, rare-earth elements (REE), Ba, Sr, Rb, K, Mg, Fe and Ca abundances (Tables 1-3). The trace and major elements were determined using the mass spectrometric isotope dilution method similar to that of Nakamura⁷ or using an electron probe microanalyser. In

addition, major-element compositions in the constituent minerals were analysed for a thin section of the bulk meteorite by an electron probe. Total blanks for all chemical processes were 0.02 ng for Rb and 0.08 ng for Sr, corresponding to <0.2% of the respective elements in most samples except for analyses of the 1 M HCl leached fraction F2-MS. The Rb-Sr data (Table 3), after corrections of the blank, are, in general, quite accurate. Details of our experimental procedures will be published elsewhere⁸.

The results for the bulk samples show that abundances of major, minor and trace elements are almost within the range of those in normal LL-chondrites. However, note that two REE patterns, normalized to the average of six normal LL-chondrites, are similar, high and slightly upwardly convex with small negative Eu anomaly, and that abundances of Ba, Rb and K are

Table 1 Elemental abundances in the Yamato-790964 LL-chondrite and the average of LL-chondrites

Element	Yamato-790964, 74		Average of LL-chondrites
	Whole rock A	Whole rock B	
Mg (%)	14.7 ± 0.1	16.1 ± 0.05	15.29*
Ca (%)	1.53 ± 0.01	1.42 ± 0.01	1.25*
K	1220 ± 3	890 ± 5	900†
Rb	3.67 ± 0.01	2.63 ± 0.05	2.5†
Sr	12.09 ± 0.02	11.8 ± 0.1	11.2†
Ba	3.35 ± 0.01	5.45 ± 0.05	3.32
La	0.348 ± 0.003	0.378 ± 0.02	0.331
Ce	0.923 ± 0.008	1.02 ± 0.02	0.865
Nd	0.695 ± 0.005	0.753 ± 0.003	0.623
Sm	0.223 ± 0.002	0.243 ± 0.003	0.200
Eu	0.0794 ± 0.0010	0.0883 ± 0.005	0.080
Gd	0.306 ± 0.005	0.326 ± 0.003	0.274
Dy	0.346 ± 0.006	0.396 ± 0.003	0.338
Er	0.236 ± 0.006	0.257 ± 0.002	0.226
Yb	0.228 ± 0.004	0.256 ± 0.003	0.232
Lu	0.0359 ± 0.007	0.0390 ± 0.006	0.0358

Abundances are expressed in p.p.m. unless otherwise stated.

* Refs 22, 23.

† Estimated from frequency distributions using data obtained in this work and from refs 9, 24. Ba and REE abundances are from refs 8, 25.

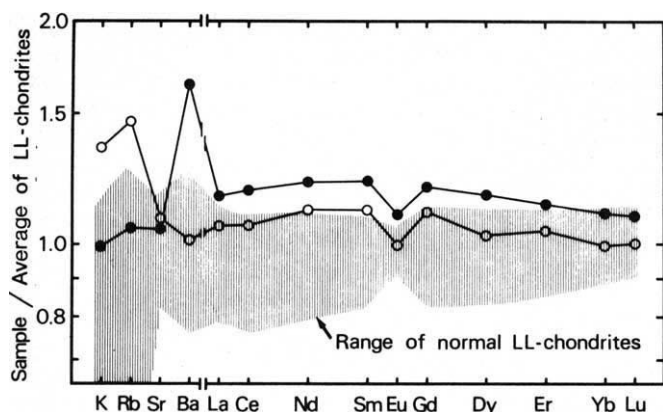


Fig. 1 Trace-element abundance pattern of the Yamato-790964 LL-chondrite. Abundances are normalized to the average of normal LL-chondrites. Note that most of the elemental abundances in the meteorite are within the range of normal LL-chondrites but show systematic fractionations in REE, Rb and K from those of normal chondrites. WR-A (○) and WR-B (●) are whole-rock samples from portions A and B, respectively.

rather variable. A similar trace-element feature has been found for other Yamato-79 unusual meteorites⁸. The variable distributions of alkali metals in LL-chondrites have also been reported for other non-Antarctic LL-chondrites^{3,9,24}.

A thin section of the meteorite reveals a heterogeneous distribution of numerous vesicles (covering ~20% in the total area) and brown glass or microcrystalline materials (~5% in the area) associated with vesicles. Euhedral, chemically zoned pyroxene ($\text{En}_{69}\text{Fs}_{24}\text{Wo}_7$ at the core, $\text{En}_{47}\text{Fs}_{15}\text{Wo}_{38}$ at the rim) and homogeneous olivine ($\text{Fa}_{29}\text{Wo}_{71}$) exist within the glass and/or microcrystalline matrix. The glasses show variable chemical compositions, ranging from 50 to 85% of normative Na-rich plagioclase ($\text{An}_{30}\text{--An}_{10}$); $\text{SiO}_2 = 45\text{--}61$, $\text{Al}_2\text{O}_3 = 23\text{--}30$, $\text{TiO}_2 = 0.2\text{--}5$, $\text{FeO} = 0.6\text{--}6.5$, $\text{MgO} = 0.2\text{--}1.6$, $\text{CaO} = 2\text{--}7.6$, $\text{Na}_2\text{O} = 4\text{--}6.5$, $\text{K}_2\text{O} = 0.1\text{--}0.2\%$. We could not recognize any particular clast but noted minor relic (radial pyroxene or barred olivine) chondrules and rounded troilite-like opaque minerals in metal grains in some areas. The general petrological features suggest that the meteorite was once heated beyond the melting point of metal/troilite, reaching the melting points of silicate minerals (1,200–1,400 °C)¹⁰ within a short time, and that melting and/or metamorphic recrystallization ensued for the whole stone, followed by rapid solidification.

These chemical and petrological features suggest that the observed variations in absolute abundances of REE, Ba, Rb and K in the bulk samples are due to heterogeneous distributions of glasses which may carry high and variable LIL trace elements. The value of ~10% for the negative Eu anomaly in both bulk samples suggests that the meteorite was related to plagioclase-controlled partial melting. For example, assuming 10–20% equilibrium partial melting of plagioclase + pyroxene (+olivine) in an LL-chondritic source, segregation of the melt from the source and addition (~10%) to a typical LL-chondrite, we would obtain 1.2–1.3 times LL-chondritic REE abundances with 10–15% negative Eu anomaly, which is similar to those of the bulk samples. Hence, the meteorite could represent an impact-produced partial melt that was removed from its point of origin and which assimilated unfractionated material before recrystallization.

In Table 3, except for the leached fraction, the olivine-rich fractions generally have larger $^{87}\text{Rb}/^{86}\text{Sr}$ ratios while the less olivine-rich and more pyroxene-rich fractions have the smaller $^{87}\text{Rb}/^{86}\text{Sr}$ ratios. However, the 1 M HCl-leached fraction (F2-MS) with highest normative olivine composition unexpectedly shows the lowest $^{87}\text{Rb}/^{86}\text{Sr}$ ratio, although the Rb and Sr concentrations are reasonable. There is no obvious explanation for this, but it is possible that low Rb/Sr component(s) were added

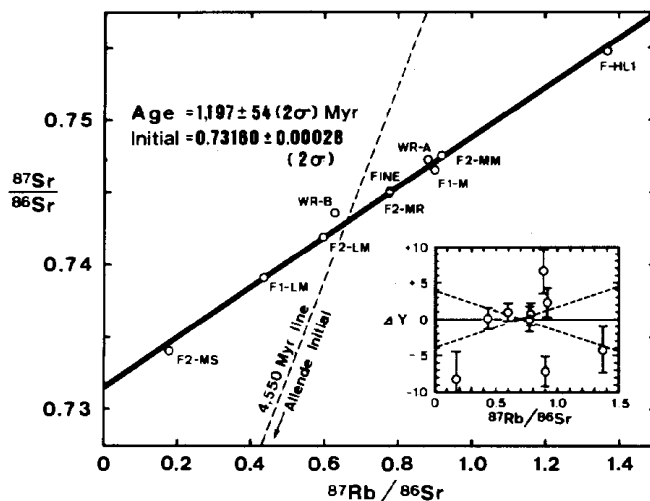


Fig. 2 ^{87}Rb – ^{87}Sr evolution diagram of the Yamato-790964 LL-chondrite. Note that data points form roughly an array but show some deviations from the regression line, suggesting the Rb–Sr isotopic system of the meteorite was almost but not completely reset by the 1,200-Myr-melting event.

to the fraction by dissolution (~18%) of the microcrystalline materials. Some minor irregularities of the Rb/Sr system are also noted among other mineral separates, indicating complex effects of Rb–Sr-rich components such as glass on the Rb–Sr systematics of the meteorite. Nevertheless, the relatively large spread of $^{87}\text{Rb}/^{86}\text{Sr}$ ratios obtained here, ranging from 0.18 to 1.4, is interesting.

In an ^{87}Rb – ^{87}Sr evolution diagram (Fig. 2), all the data points obtained roughly show an array. However, the data point of whole rock B deviates markedly from the general trend. Excluding whole rock B, nine data points define a best-fit linear regression line, which yields an age of $1,197 \pm 54$ (2 σ) Myr and an initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of 0.73160 ± 0.00028 (2 σ). Despite a large range of values of $^{87}\text{Sr}/^{86}\text{Sr}$ among the mineral separates, the errors in age and initial ratio obtained here are large. This is apparently caused by dispersion of some data points beyond analytical uncertainties, as shown in the inset to Fig. 2. Note that three samples (F2-MS, F2-MR and F-HL1) were treated with ≤ 1 M HCl and Clerici's solutions for mineral separation and washing. Although there are minor analytical problems with these samples a close examination of the analytical data suggests that a possible selective leaching of Rb to Sr is not too serious. Also we must consider the potential importance of a weathering effect on the trace-element behaviour in Antarctic samples¹¹ when evaluating such problems. However, since the specimens analysed in this work were sampled from the inner part of the meteorite and appeared quite fresh under the binocular microscope, the observed isotopic disturbances are considered to be real. Specifically, deviations of data points of whole rock (WR) A, B and F1-M are the most significant.

Because the weighted mean ratios of $^{87}\text{Rb}/^{86}\text{Sr}$ and $^{87}\text{Sr}/^{86}\text{Sr}$ of mineral separates are similar to those of whole rock A, this rock may roughly represent portion A of the meteorite in terms of Rb–Sr systematics, but not necessarily the total meteorite. Note that the Rb/Sr data of whole rock A ($^{87}\text{Rb}/^{86}\text{Sr} = 0.881$), as well as those of whole rock B ($^{87}\text{Rb}/^{86}\text{Sr} = 0.629$), fall off significantly from the 4,500-Myr evolution line, suggesting that the meteorite was subjected to severe Rb/Sr fractionation at the time of the 1,200-Myr thermal event. Assuming a two-stage evolution, formation of the meteorite parent body with the same initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio as the Allende meteorite (bias-adjusted value = 0.69884)¹² at 4,500 Myr ago, and Rb/Sr fractionation at 1,200 Myr ago, the $^{87}\text{Rb}/^{86}\text{Sr}$ ratio of the meteorite at the time of formation is calculated to be 0.67. This value is 24% smaller than that of whole rock A but is similar to the $^{87}\text{Rb}/^{86}\text{Sr}$ ratio

Table 2 Major element and estimated mineral composition for specimens from the Yamato-790964 LL-chondrite

Sample	MgO (%)	FeO (%)	CaO (%)	Ol (%)	Opx (%)	Cpx (%)	Gls (%)	Other (%)
Whole rock								
WR-A Chip from portion A	24.4	—	2.14					
WR-B Chip from portion B	27.7	—	2.00					
Mineral separates from portion A								
<300 mesh								
Fine Suspended in acetone	—	—	—					
F1-LM Less magnetic	21.8	18.1	3.23	25	18	35	5	17
F1-M Magnetic	25.1	22.4	1.57	54	11	11	7	17
300 < <200 mesh								
F2-LM Less magnetic	25.2	18.1	3.52	28	25	37	5	5
F2-MB Magnetic (bulk)	27.5	22.4	1.51	63	8	11	3	15
F2-MR Magnetic (residue)*	21.8	14.5	3.37	11	37	35	11	6
F2-MS Magnetic (leached)*	31.4	27.8	0.34	(83	=0	=0	=0	18)
F2-MM More magnetic	26.3	22.8	1.63	56	12	10	5	17
F-HL1 Density-separate†	31.3	26.4	0.93					

Major elements were determined by an electron probe microanalyser for 100–300 grains from the mineral separates except for samples of WR-A, WR-B, F2-MS and F-HL1, which were analysed by isotope dilution. Mineral compositions were estimated from the distribution of the major-element composition for each grain. Ol, Olivine; Opx, orthopyroxene; Cpx, clinopyroxene; Gl, glass; other, microcrystalline materials + metal + troilite.

* Soluble (F2-MS) and insoluble (F2-MR) fractions obtained by leaching F2-MB with 1M HCl for 80 h at room temperature.

† Separated by Clerici's solution ($3.37 < \text{density} < 3.45 \text{ g cm}^{-3}$) and washed with dilute HCl before analysis.

of most LL-chondrites^{9,24}. Therefore, we suggest that the meteorite had a typical LL-chondritic Rb/Sr ratio between 4,500 and 1,200 Myr and that an enrichment of Rb relative to Sr occurred 1,200 Myr ago. Such a Rb/Sr fractionation is in accordance with the melting-assimilation model deduced from the REE data mentioned earlier, and is also consistent with the trace-element data for the associated meteorites⁸. However, the inconsistent behaviour of Rb/Sr with the REE which is observed for whole rock B, and the lack of complete isotopic equilibration of both whole rocks (A and B shift to the left of 1,200-Myr isochron) indicate that the effects of the 1,200-Myr thermal event on the Rb/Sr systematics of the meteorite were very complex. Considering such isotopic evidence, together with the REE, Ba and K data, we suggest that the chemical and petrological characteristics of the meteorite are best explained as the result of intense impact(s) which yielded partial melting and assimilation of the regolith on the LL-chondrite parent body $\leq 1,200$ Myr ago.

It is interesting that the 1,200-Myr age obtained for the Yamato-790964 meteorite is the youngest so far reported for impact-produced lithic materials in brecciated meteorites. Most such meteorites show significantly older ages ($> 3,400$ Myr)^{12–14}

with the exception of the H-clast in the St Mesmin LL-chondrite which shows a 1,400-Myr K–Ar age¹⁵. Moreover, except for SNC meteorites, the Yamato-790964 LL-chondrite would be the first complete stone to show the latest crystallization age.

Because no chemical and petrological similarities seem to exist between the LL-related chondritic material mentioned above and SNC meteorites, a possible genetic relation between the two may be ruled out. The 1,300-Myr age obtained for SNC meteorites^{16–21} has been explained as a crystallization age which marks extensive volcanic activity on a well-differentiated planet such as Mars^{20,21}. On the other hand, the 1,200–1,400 Myr age obtained for the Y-790964 and St Mesmin meteorites is interpreted as signifying a time of strong impact activity on the LL-chondrite parent body (asteroidal planet). How are these facts related? We are increasingly puzzled that all the major young perturbing events observed for meteorites have an age close to 1,200–~1,400 Myr. As the number of results increases, one should examine whether this is a real effect of a major impact-related event on meteorite parent planets or a sampling bias. In the former case, like the 4,000-Myr lunar cataclysm, one should look for collision and storage processes lasting for billions of years.

In any case, in view of the large amount of the similar types of meteorite found in the Yamato Mountain area, Antarctica, we suggest that the 1,200-Myr age obtained here is a non-trivial marker in the evolution of meteorites.

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Table 3 Results of Rb–Sr analyses for the whole rocks (WR) and mineral separates from the Yamato-790964 LL-chondrite

Samples	Weight (mg)	Rb (p.p.m.)	Sr (p.p.m.)	⁸⁷ Rb/ ⁸⁶ Sr	⁸⁷ Sr/ ⁸⁶ Sr
WR-A	52	3.669	12.09	0.881	0.747205 ± 128
WR-B	50	2.559	11.82	0.6285	0.743553 ± 53
Fine	48	3.229	12.02	0.7802	0.745024 ± 32
F1-LM	12	1.517	10.10	0.436	0.739087 ± 59
F1-M	76	3.475	11.19	0.902	0.746516 ± 44
F2-LM	21	2.117	10.28	0.598	0.741913 ± 30
F2-MR	29	7.046	26.33	0.777	0.744918 ± 32
F2-MS	75*	0.0433	0.705	0.178	0.734080 ± 250
F2-MM	54	4.171	13.20	0.9178	0.747503 ± 50
F-HL1	51	2.297	4.90	1.363	0.754679 ± 68
NBS 987 standard (mean of 10 runs)				0.710212 ± 21†	

Isotopic analyses were carried out at Okayama University using a mass spectrometer model MAT-261. Precisions for ⁸⁷Rb/⁸⁶Sr are estimated to be better than 0.8% except for the F2-MS. ⁸⁷Sr/⁸⁶Sr ratios are normalized to ⁸⁶Sr/⁸⁸Sr = 0.1194. Errors correspond to last digits and are at the 95% confidence limit.

* Calculated weight which contains 10% uncertainty.

† Ratio is in agreement with our previous analysis¹⁹.

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Melting of peridotite to 14 GPa and the genesis of komatiite

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It is generally accepted that the Earth's upper mantle is made of peridotite^{1,2}. In volatile-absent conditions, partial melting of mantle peridotite close to the solidus yields a variety of basaltic melts that are progressively more olivine-normative with increasing depth^{3,4}. At 3–4 GPa (30–40 kbar), the highest pressures so far investigated, the melts are picritic with up to 20 wt% MgO (ref. 5). Basaltic magmas that are dominant on the Earth's surface today, therefore, are considered to have been generated at relatively shallow depths (<100 km)^{1–6}. Here we extend the pressure range of the melting experiments to 14 GPa and show that melts close to the peridotite solidus at 5–7 GPa are no longer basaltic but are komatiitic with >30 wt% MgO. Based on these results, we describe a model for the genesis of komatiite magmas by partial melting at depths of 150–200 km in the Archaean upper mantle.

The starting materials for the experiments were two lherzolites (Table 1). KLB-1 is a protogranular spinel lherzolite from Kilbourne Hole⁷, New Mexico, and PHN1611 is a sheared garnet lherzolite from Thaba Putsoa⁸, Lesotho. Both may represent undepleted mantle material and are close to pyrolite-III⁹ in composition. Garnet lherzolite PHN1611 has been previously investigated at pressures ≤ 3.5 GPa (refs 10–13).

Crushed powders (<5 μm) were dried at 1,000 °C at the NNO (Ni–NiO) buffer. Wire loop experiments¹⁴ were performed at 1 atm in a controlled CO₂–H₂ atmosphere close to the NNO buffer. Experiments at 1.5 and 3 GPa were carried out with a piston-cylinder apparatus¹⁵ using a 0.5-inch talc–Pyrex assembly. Run conditions, methods and procedures are similar to those described in ref. 5.

At 5 GPa and above, a split-sphere apparatus¹⁶ at Okayama University was used. Up to 7.5 GPa, a graphite furnace assembly was inserted into a pressure medium of semi-sintered MgO in the shape of an octahedron with an 18-mm edge length. Above 7.5 GPa, LaCrO₃ was used as the furnace element in a 14-mm-edge octahedron of MgO (Fig. 1a). Temperatures were monitored with W–Re thermocouples and have uncertainties of

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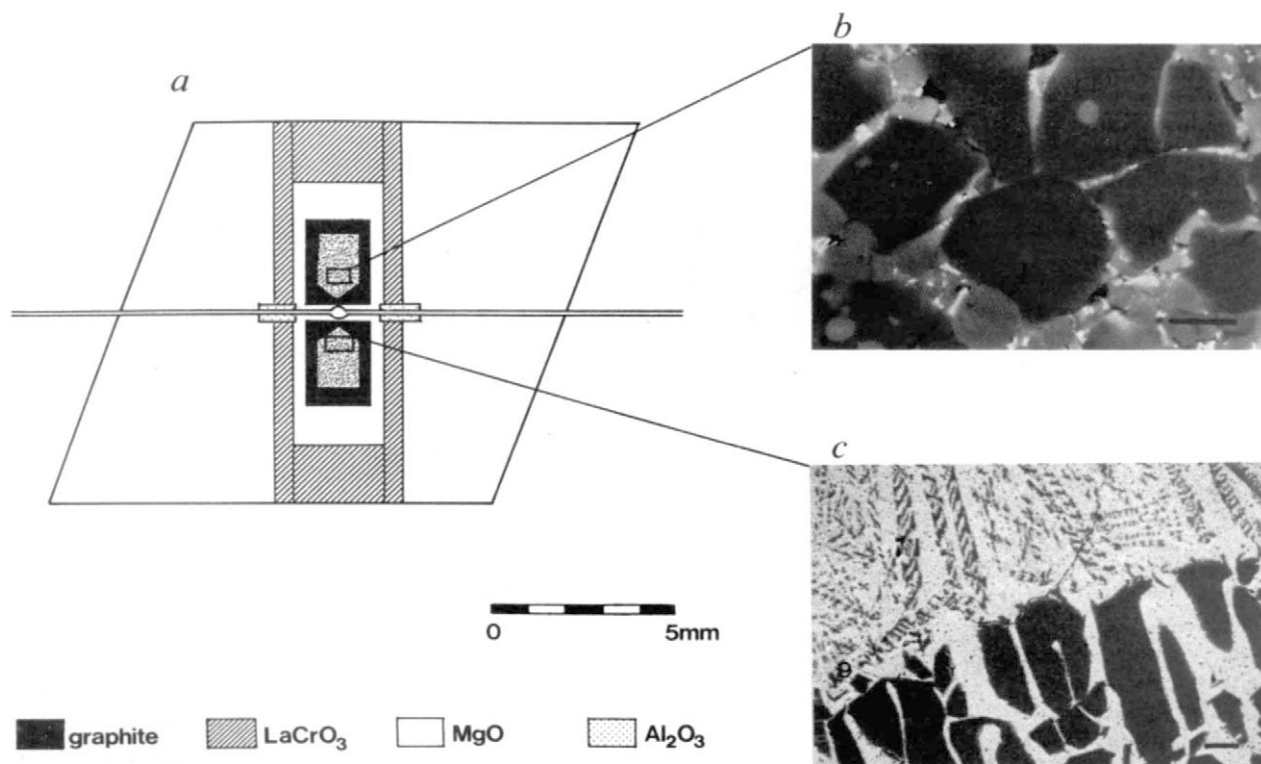


Fig. 1 *a*, Cross-section of the MgO pressure medium with the furnace assembly, thermocouple and sample in place. This design was used at pressures ≥ 7.5 GPa; below 7.5 GPa a graphite furnace with a fired pyrophyllite sleeve was used. *b*, Back-scattered electron image of the charge from an experiment at 7.5 GPa and 1,900 °C. The melt (now consisting of white areas of iron-rich quench pyroxenes) occurs in pockets between olivine (large dark grains) and garnet (small grey grains). *c*, Back-scattered electron image of the charge from an experiment at 5 GPa and 1,800 °C. The segregated melt is seen at the top with coexisting olivine crystals below the interface. The melt region shows abundant well-developed quench olivine and pyroxene. Scale bars in *b* and *c*, 10 μm .

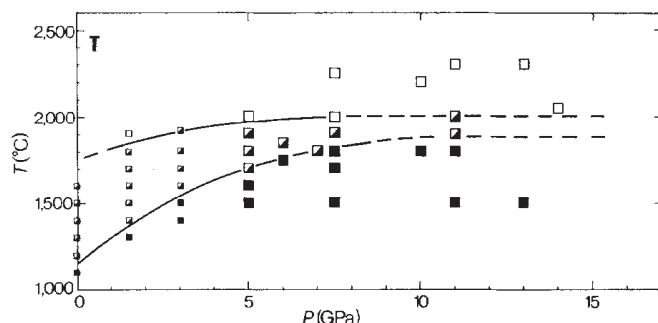


Fig. 2 Phase diagram for Iherzolite KLB-1. Results from 1 atm and piston-cylinder experiments indicated by small squares; results from split-sphere experiments indicated by larger squares. ■, Subsolidus; ■, crystals + liquid; □, superliquidus.

$\pm 50^\circ\text{C}$ due to the temperature gradient within the furnaces¹⁷. Pressures were calibrated at several fixed points at room temperature [Bi(I-II), Bi(III-V) and Pb(I-II)] and at high temperatures (quartz-coesite, fayalite- γ spinel, coesite-stishovite and forsterite- β spinel). Uncertainties in pressures are estimated to be <0.05 GPa up to 3 GPa, and 0.5 GPa above 3 GPa. Experiments above 5 GPa were typically 10 min in duration.

Figure 2 shows the solidus and liquidus curves for KLB-1. At 1 atm the partial melt region is $\sim 600^\circ\text{C}$, whereas it contracts to $\sim 100^\circ\text{C}$ at 10 GPa as predicted by Herzberg¹⁸. Olivine is in the liquidus phase at all pressures. The second liquidus phase, however, changes with increasing pressure: chromian spinel (1 atm), Ca-poor orthopyroxene (0.5–3 GPa), pigeonitic clinopyroxene (3–5 GPa) and pyrope-rich garnet (5–11 GPa). Figure 1b shows the distribution of melt coexisting with olivine and garnet in an experiment at 7.5 GPa and $1,900^\circ\text{C}$. The melt is confined to small isolated pockets in triple junctions between the crystals. Figure 1c shows melt, which has segregated from the matrix, coexisting with olivine only at 5 GPa and $1,800^\circ\text{C}$. In experiments using the split-sphere type apparatus, segregation of the partial melts during short runs was probably due to the thermal gradient and/or stress gradient in the furnace assemblies.

The chemical compositions of partial melts formed within 50°C of the dry solidus are shown in Table 1. To avoid the modification of melt compositions, during quenching^{5,19}, only segregated partial melts were chemically analysed. Because high-MgO melts do not quench to a glass, but crystallize with well-developed spinifex textures (Fig. 1c), a defocused electron beam was used for their analysis and the compositions of quench

crystals in an area $>100\ \mu\text{m}$ were averaged. The melts become increasingly MgO-rich or olivine-normative as pressure increases, and all melts formed above 5 GPa may be classified as peridotitic komatiite in terms of their high MgO content and the chondrite-like abundance of CaO and Al_2O_3 (refs 20, 21). Melts near the solidus at ~ 5 GPa contain ~ 30 wt% MgO and are very close to the primary komatiite magma composition estimated by Arndt *et al.*²² (Table 1).

Preliminary results for PHN1611 are similar, although because of lower MgO/FeO and higher $\text{Na}_2\text{O} + \text{K}_2\text{O}$ content, the solidus is displaced to slightly lower temperatures. When compared at the same pressure (P) and temperature (T), partial melts from PHN1611 are enriched in FeO and higher in $\text{CaO}/\text{Al}_2\text{O}_3$ than those from KLB-1 (Table 1).

The origin of komatiite magmas has been discussed by several authors^{6,20–28}. Komatiite has an unusually high liquidus temperature ($>1,650^\circ\text{C}$)²³ at 1 atm and at low pressures. A very high degree of partial melting (50–70%) of peridotite is required to form a komatiitic liquid^{6,21,23}. Both theoretical^{29,30} and experimental observation²⁵ indicate, however, that partial melt will segregate from the peridotite matrix instantly when the degree of partial melting exceeds a critical value which lies between 10 and 30 vol.%. Thus, it is unlikely in static conditions that a very high degree of partial melting can be achieved within the Earth.

A multistage melting model has been proposed by Arndt²⁵ in which komatiitic magma is formed in a rising diapir after several cycles of partial melting and melt extraction. The possibility of catastrophic magma genesis induced by impacts of large meteorite bodies has also been proposed²⁴. Furthermore, a deep-seated magma ocean in the Archaean mantle has been invoked²⁶ to explain komatiite petrogenesis.

On the basis of the present experimental results, we propose a simple model (Fig. 3) for the derivation of komatiite magma by partial melting of a rising peridotite diapir as it intersects the mantle solidus at a depth of 150–200 km (path A–B). Because initial melts at these depths have komatiitic compositions, it is possible that komatiitic melts segregate and ascend to the surface (path B–C) before the degree of partial melting of the source region reaches a high percentage. Similar models have been proposed for the genesis of picritic (path D–E–F) and basaltic (path G–H–I) magmas at shallower depths³ and the possibility of komatiite magma generation by partial melting of garnet peridotite at very high pressures (>5 GPa) has already been proposed^{27,28}.

Because melts of komatiite composition are among the highest temperature magmas known, they may be derived from the deepest levels of magma generation. These depths may be limited

Table 1 Chemical compositions of the starting materials and partial melts

	Starting materials			Peridotite partial melts						Average peridotitic komatiites		
	KLB-1	PHN1611	Pyrolite	P (GPa) T (°C)	($\times 10^{-4}$) 1,200	3.0 1,550	5.0 1,700	6.0 1,850	6.0* 1,850	7.0 1,800	MgO > 30 wt%	< 30 wt%
SiO ₂	44.48	44.54	45.20		54.2	46.9	47.2	46.3	45.8	48.0	44.9	46.0
TiO ₂	0.16	0.25	0.71		0.65	0.92	0.15	0.18	0.38	0.11	0.19	0.32
Al ₂ O ₃	3.59	2.80	3.54		15.1	11.0	5.1	4.8	4.5	3.7	5.3	7.4
FeO(t)	8.10	10.24	8.47		5.6	7.8	9.3	8.9	11.4	7.8	10.4	11.5
MnO	0.12	0.13	0.14		0.09	0.20	0.12	0.17	0.20	0.13	0.18	0.22
MgO	39.22	37.94	37.48		8.4	19.2	31.3	32.9	31.3	35.3	33.6	26.5
CaO	3.44	3.32	3.08		12.3	12.2	4.8	4.4	4.6	3.1	5.0	7.4
Na ₂ O	0.30	0.34	0.57		2.05	1.20	0.49	0.46	0.52	0.26	0.35	0.45
K ₂ O	0.02	0.14	0.13		ND	ND	ND	ND	ND	ND	0.08	0.10
P ₂ O ₅	0.03	ND	0.06		ND	ND	ND	ND	ND	ND	ND	ND
Cr ₂ O ₃	0.31	0.29	0.43		0.14	0.43	0.46	0.41	0.37	0.41	0.26	0.39
NiO	0.25	ND	0.20		ND	ND	ND	ND	ND	ND	0.02	0.01
Total	100.02	99.99	100.01		98.5	99.9	98.9	98.5	99.1	98.8	100.28	100.29
				Fo ^{oliv}	89.9	92.6	94.1	94.4	92.8	—		
				K _D (Fe/Mg) ^{oliv} _{liq}	0.30	0.35	0.38	0.39	0.38	—		

Analysis for KLB-1 by H. Haramura. Compositions of PHN1611, pyrolite-III and peridotitic komatiite are from refs 8, 9, 22. Partial melts were analysed by EPMA (electron probe microanalyser) using a defocused $50\text{-}\mu\text{m}$ beam diameter; the coexisting olivines were analysed using a focused-beam, 15-kV accelerating voltage, beam current of 2.0×10^{-8} A and counting times of 40–70 s.

* Peridotite partial melt formed from PHN1611.

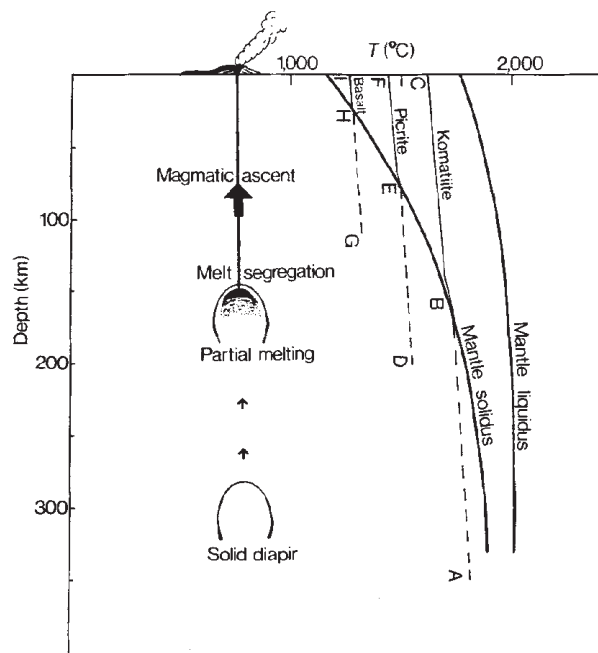


Fig. 3 Diapiric model for the generation of komatiite magma at depths of 150–200 km.

by the density crossover between komatiite melts and coexisting residual mantle solids^{28,29}. Komatiite magmas are not common in the Phanerozoic²¹, presumably either because the temperature in the Archaean upper mantle was higher than in more recent geological time, or because diapirs originated from greater depths in the Archaean.

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Preparation and catalytic properties of ionic sodium clusters in zeolites

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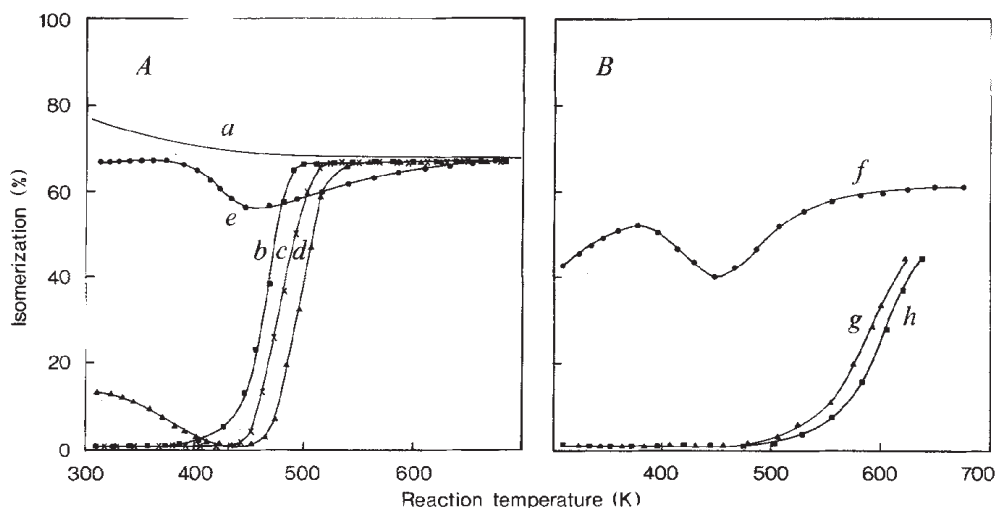
Exposure of high-alumina zeolites to sodium metal vapour produces coloured centres^{1–5} which have been associated with the formation of ionic sodium clusters, located in the zeolite cages. To form these centres, excess alkali metal is required, which contaminates the reaction vessel and makes the system unsuitable as a heterogeneous catalyst. The pioneering work of Pines⁶ in the area of basic catalysis on sodium metal deposited on alumina implies that ionic clusters in zeolites should exhibit interesting catalytic properties, but there have been no reports of such activity because the procedure yields a material which is difficult to reproduce. Here we present a new method for the preparation of such clusters in zeolites, consisting of the impregnation of a dehydrated zeolite with alcoholic solutions of sodium azide, followed by the controlled decomposition of the azide. As demonstrated by electron-spin resonance (ESR) spectroscopy, ionic sodium clusters are formed in the zeolite pores. These clusters exhibit catalytic properties in both isomerization and hydrogenation reactions of alkenes and alkynes. We believe that this represents the first realistic method for the preparation of alkali metal clusters in zeolites as well as the first catalytic characterization of such systems.

In the present experiments we set out to develop a reliable method for the preparation of ionic sodium clusters in zeolites and to determine the catalytic properties of such material. The preparation method followed Fejes *et al.*^{7–9} who reported that the residual acidity of Y zeolites, existing after cation exchange with Na⁺ ions, could be neutralized on heating a physical mixture of the zeolite with small amounts of sodium azide (amounts up to 1.12 mmol of NaN₃ per g of zeolite Y were used). We found, however, that when increasing quantities of this product are impregnated on the zeolites A, X and Y using the specific procedure outlined below, this zeolite is turned gradually into a very efficient basic heterogeneous catalyst.

Typically, to 1 g of NaY zeolite present in the form of powder or as 0.8–1-mm agglomerates, and dried at 723 K under a flow of inert gas, was added under inert atmosphere a slurry containing 3.85 mmol of sodium azide and 9.12 mmol of methanol. The volume of this slurry is just sufficient to fill the zeolite pore volume. This wet cake was transferred to an ESR cell or a tubular flow reactor. The water-free cake was first activated *in situ* at a temperature of 523 K and then at 673 K, while the system was continuously purged with an inert gas. In the tubular reactor, the following catalytic conversions were followed: geometrical and double-bond isomerization of *cis*-2-butene and hydrogenation of acetylene, benzene and *cis*-2-butene. The ratio of the product isomers in the isomerization of *cis*-2-butene can be used as a criterion to determine whether the reaction is acid or base-catalysed^{6,10–14}.

Figure 1A illustrates how the isomerization activity of NaY first declines on addition and decomposition of increasing amounts of NaN₃, and then increases. When an average of eight molecules of NaN₃ are impregnated per supercage of zeolite (3.85 mmol per g of zeolite Y), an activity pattern with two maxima is obtained (Fig. 1B). The catalysts with an activity maximum at low temperature have a bright-red colour and exhibit an ESR spectrum having 13 lines with $g = 2.003$ and $a = 3.35 \pm 0.10$ mT (Fig. 2A). This spectrum has been ascribed to an ionic Na₄³⁺ cluster². The cluster signal is accompanied by a single broad line at $g = 2.078$. This signal has not been reported in previous work and is, for the moment, assigned to large alkali metal particles located in the zeolite supercages or at the outer surfaces of the zeolite crystals. On thermal sintering, a single line at $g = 2.003$ appears (Fig. 2B); this signal is close to that

Fig. 1 Isomerization of *cis*-2-butene over dehydrated NaY zeolites: *A*, impregnated with increasing amounts of NaN₃, using a volume hourly space velocity (VHSV) of 36, and *B*, impregnated with 3.85 mmol of azide per g of zeolite at a VHSV of 100. *a*, The thermodynamically expected equilibrium conversion; *b*, the isomerization activity of the parent NaY and its activity after addition of *c*, 1.54; *d*, 2.88; and *e* or *f*, 3.85 mmol of azide per g of zeolite; *g*, after thermal treatment of *f* at 700 K; *h*, after treatment of *f* with oxygen at room temperature and heating in an inert atmosphere at 673 K.



reported for bulk metal⁵. Extended sintering ultimately causes the disappearance of the signal ascribed to the ionic cluster. During this treatment, the broad line at $g = 2.078$ is unaffected while the isomerization activity varies in parallel with the intensity of the signal, ascribed to the existence of the ionic cluster. On exposure to oxygen of the red catalyst (at room temperature) its colour, low-temperature catalytic activity and the 13-line ESR spectrum vanish instantly. The disappearance of the other ESR signals requires longer exposure times.

The initial 1- to *trans*-2-butene product ratios from *cis*-2-butene at low conversions are given in Table 1. The values of these ratios increase in parallel with the amount of NaN₃ added to zeolite Y. If the initial 1-butene to *trans*-2-butene ratios are >2 , the *cis*-2-butene isomerization is catalysed by basic sites^{6,10-14}. This behaviour confirms that on addition of increasing amounts of NaN₃ to NaY zeolite, residual acidity first has to be neutralized and eventually basic catalytic centres form. We associate this high base-catalysed activity with the presence of the ionic sodium clusters. The residual activity observed after exposure of the catalyst to oxygen is associated with the sodium metal particles located either in the supercages of the Y zeolite or at the external surface of the zeolite crystals. The presence of ionic clusters, as evidenced by the 13-line ESR spectrum and the high catalytic activity, is extremely sensitive to the presence of residual oxygen and water molecules. Therefore, the catalytic sites are definitely sodium clusters and not sodium oxide or hydroxide species which are dispersed in the zeolite cages.

The 13-line ESR spectrum was observed by Harrison *et al.*⁵ only for zeolites containing sodalite units on exposure to sodium

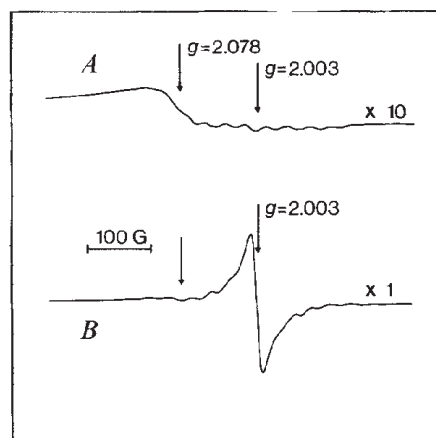


Fig. 2 ESR signals from dehydrated NaY after impregnation with 3.85 mmol NaN₃ per g of zeolite and after subsequent thermal activation (*A*) or after sintering of *A* in an inert atmosphere at 673 K (*B*).

metal. They concluded, therefore, that the sodium ionic clusters are located in the hidden cages of these zeolites. At this stage, it is not clear how electron transfer from the hidden ionic clusters to the butene molecules occurs, or how this view can be reconciled with the high catalytic activity, but we have ample evidence that the high-base catalysed activity in the butene isomerization appears in parallel with these ionic clusters.

Table 2 shows that the catalysts containing ionic clusters are also able to hydrogenate butene and acetylene at non-negligible rates, while benzene in the same conditions cannot be hydrogenated. Peculiar properties of these catalysts are: (1) 50% of the products obtained from *cis*-2-butene, are saturated (butane); the other 50% are feed isomers. This indicates that hydrogenation as well as isomerization occurs at comparable rates. (2) Hydrogenation of acetylene on these basic centres is highly selective as the ethylene/ethylene + ethane ratio is close to 85%.

When the NaN₃ method is used with zeolite X, an intense blue colour, a 19-line ESR spectrum associated with a Na₆⁵⁺ cluster¹ and a pronounced low-temperature catalytic activity is found. To a minor extent, this is also true for zeolite A, while with other zeolite supports such as Na-ZSM-5 and mordenite, no colour is observed, nor is an ESR signal with $g = 2.003$ detected. Such samples also show no low-temperature basic activity.

In this respect, we have been unable to reproduce the catalytic activity of mordenite containing only Na⁺ ions in hydrogenation reactions, as reported by Minachev¹⁵. These results confirm that either the ionic clusters are stabilized in the sodalite cages as suggested by Harrison *et al.*⁵, or they are not formed in high-silica zeolites, so the virtue of A, X and Y zeolites may be the close

Table 1 Initial ratios (*R*) of 1-butene to *trans*-2-butene from *cis*-2-butene at low conversions (~1%) and low reaction temperature (325 K)

Catalyst*	<i>R</i>
<i>a</i>	0.05
<i>b</i>	0.98
<i>c</i>	0.83†
<i>d</i>	2.63†
<i>e</i>	8.65‡

* Catalysts of Fig. 1. † VHSV = 36. ‡ VHSV = 250.

Table 2 Hydrogenation activity of NaY zeolites containing initially 3.85 mmol NaN₃ per g of zeolite

Reaction	VHSV	Reaction temperature (K)	% Conversion
Hydrogenation butenes	125	348	36
Hydrogenation acetylene	80	343	10
Hydrogenation benzene	76	345	0

presence of several acid sites, due to the low Si/Al ratio, which can form a suitable counterion to the ionic cluster Na_4^{3+} . In view of the latter possibility, it is no longer necessary to assume the presence of such clusters in hidden locations and direct contact of the reacting hydrocarbons is possible with these clusters.

We have shown that on impregnation of appropriate quantities of NaN_3 into the high-alumina zeolites A, X and Y, and on its subsequent thermal decomposition in the specific conditions described, ionic sodium clusters are formed which exhibit typical basic catalytic properties at low reaction temperatures.

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Novel pathway connecting the outer and inner vertebrate retina

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In many fish retinas, thin axons from the external horizontal cells extend through the inner nuclear layer and expand into large terminal processes that lie along the border of the inner nuclear and inner plexiform layers¹. Although the horizontal-cell axon terminals are structurally very prominent, their function is unknown². Here we report morphological and functional evidence that signals from catfish (*Ictalurus punctatus*) horizontal-cell axon terminals can be transmitted directly to amacrine cells. Current injected into horizontal-cell axon terminals produces responses from both transient and sustained amacrine cells very similar to those elicited by light stimuli. Electron microscope observations show chemical synapses from the axon terminals onto amacrine cell perikarya and processes. These data suggest that amacrine cells in the catfish retina receive two inputs, one from bipolar cells and the other from horizontal-cell axon terminals.

For electron microscopy, standard fixation and staining procedures were used³. Fourteen sets of serial sections (200-400 sections per set) from five retinas were examined. The horizontal-cell axon terminals are easily recognized in the electron microscope because of their size and the abundance of microtubules⁴. We observed typical chemical synapses made by the terminals onto postsynaptic elements in the inner nuclear layer and along the border of the inner plexiform layer. Approximately two-thirds of the postsynaptic elements were identified as amacrine cell processes or perikarya because they contained pale cytoplasm and scattered synaptic vesicles and, occasionally, made conventional synapses. Furthermore, serial section tracing did

not reveal any synaptic ribbons in the postsynaptic elements, or processes associated with these elements which extended towards the outer plexiform layer. Figure 1 shows an example of a horizontal-cell axon terminal synapsing onto the cell body of a presumed amacrine cell in the inner nuclear layer. The synapse is characterized by an aggregation of round synaptic vesicles associated with increased density on both the pre- and postsynaptic membranes. A subsurface cistern frequently occurred along the postsynaptic site. One such synapse was found, on average, in each $60\text{-}\mu\text{m}^2$ area of contact made by the horizontal-cell axon membrane with the amacrine cell perikarya and elements in the inner plexiform layer. We have so far observed a total of 18 such contacts, 2 on cell perikarya and 16 on processes.

For physiological experiments, extrinsic current was injected into horizontal-cell axon terminals in the eyecup preparation while recordings were made from amacrine cells. Two intracellular electrodes with tips separated by $\sim 0.4\text{ mm}$ were used; one electrode injected current into the horizontal cells while the other recorded responses from amacrine cells. In the catfish retina only one type of horizontal cell receiving inputs from cones has been reported; its perikaryal response shows a monophasic receptive field of $\sim 0.5\text{ mm}$ in halfwidth and its axon-terminal response shows a receptive field with a halfwidth of $\sim 1\text{ mm}$ (refs 5, 6). This difference in receptive-field size, which may be demonstrated by either a travelling or random bar of light⁶, enabled us to identify unambiguously the site of recording. Two types of amacrine cell response can be identified in the catfish retina; one type produces a sustained response and is referred to as sustained or type-N amacrine cell and the other produces a transient response and is termed transient or type-C amacrine cell⁷⁻⁹.

We performed current injection experiments in six retinas and observed that current injected into the horizontal-cell axon terminals always produced responses in both the transient (15 pairs) and sustained (15 pairs) types of amacrine cells⁸⁻¹⁰. Examples are shown in Fig. 2 in which responses from both types of cells were produced by both light and current injected into the horizontal-cell axon terminal. Records *a* and *b* were from a depolarizing sustained (type-NA) amacrine cell

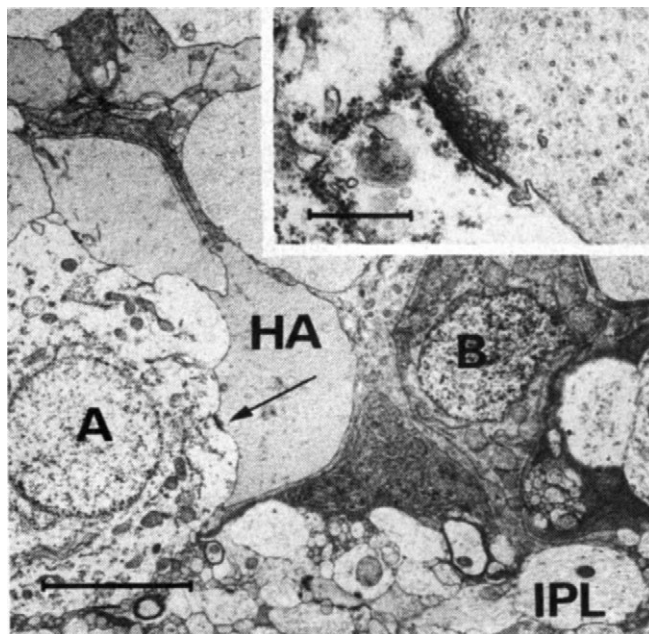


Fig. 1 Electron micrograph of cross-section of a catfish retina showing a synapse from a horizontal-cell axon onto the amacrine-cell perikaryon. Arrow, synapse; A, amacrine cell; HA, horizontal-cell axon; B, bipolar cell; IPL, inner plexiform layer. Scale bar, $5\text{ }\mu\text{m}$. Inset, high magnification of the synapse indicated by the arrow. Scale bar, $0.5\text{ }\mu\text{m}$.

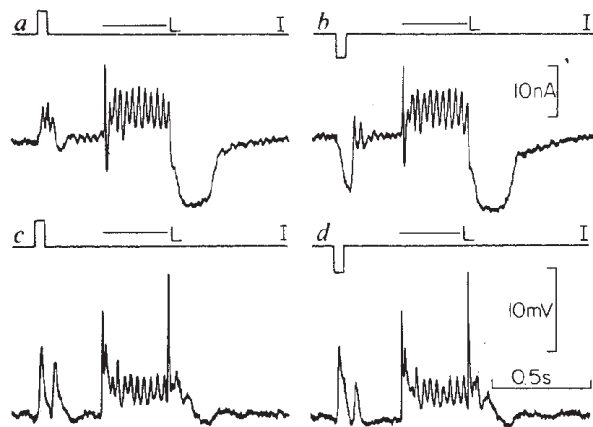


Fig. 2 Responses recorded from sustained or type-N (*a, b*) and transient or type-C (*c, d*) amacrine cells. The responses are typical of catfish amacrine cells as reported previously^{7,8}. Responses were evoked by a current (*I*) pulse followed by a light flash, the approximate timing of which is shown by the bar marked *L*. Quantitative analyses of latency of current or light responses have been described elsewhere⁶. The type-N cell shown is the depolarizing variant (type NA) but current injected into the horizontal-cell axon produced similar responses (with the polarity of slow potential reversed) from the hyperpolarizing variants (type NB). Note that, in the type-N cell, the polarity of the current-induced slow potentials reversed when the polarity of current was reversed. Oscillations were superposed on the depolarizing phase of the response. Some type-C cells produced oscillations as seen in *c, d*, but some did not. Calibrations apply to all records.

whose light-evoked responses were characterized by an initial depolarizing transient followed by oscillatory wavelets. There was also a very large afterhyperpolarization at the cessation of illumination. A depolarizing current injected into the axon terminal (Fig. 2*a*) also produced a depolarizing response on which a few oscillatory wavelets were seen, whereas a hyperpolarizing current (Fig. 2*b*) produced a hyperpolarizing response with wavelets seen on the off-depolarization. Light-evoked responses from a transient (type-C) cell are shown in Fig. 2*c, d*. Illumination elicited large on- and off-transient depolarizations with oscillations between, whereas depolarizing (Fig. 2*c*) and hyperpolarizing currents (Fig. 2*d*) both produced on- and off-transient depolarizations.

There are three possible pathways by which the horizontal-cell axon terminals can communicate with amacrine cells: (1) via horizontal-cell perikarya which synapse onto the bipolar cells and then contact amacrine cells; (2) via horizontal-cell axon terminals synapsing onto the bipolar cells which contact amacrine cells; or (3) via synapses directly onto the amacrine cells. The first and second possibilities seem unlikely because: (1) current (≤ 40 nA) injected into axon terminals failed to evoke a response in bipolar cells (10 pairs), whereas current (< 10 nA) injected into horizontal-cell perikarya always evoked responses from bipolar cells (32 pairs); (2) current (≤ 40 nA) injected into the axon terminals produced only very small responses (0.7 mV) in the horizontal-cell perikarya (12 pairs)⁶; and (3) current of < 10 nA injected into the axon terminals produced responses from the amacrine cells (Fig. 2). Furthermore, axon terminals have not been observed to synapse onto the bipolar cells in catfish, whereas synapses made by the axon terminals onto the amacrine cells have been found.

We conclude that there are two independent pathways for signal transmission from the outer to inner plexiform layers in the catfish retina. One is the established pathway through bipolar cells and the other is a new pathway through the horizontal-cell axon terminals. Two recent anatomical studies have also identified synapses made by horizontal-cell axonal processes in goldfish (ref. 11 and D. W. Marshak and J. E. Dowling, personal communication); hence such a synaptic pathway may not be unique to the catfish.

Note that the current-evoked responses elicited in the ama-

crine cells have similar characteristics to those of the light-evoked responses, showing that complex responses such as oscillations in the type-N cell and transient depolarizations in the type-C cell are produced when signals are transmitted directly from the axon terminals to the amacrine cells. This suggests that the complex responses of amacrine cells reflect intrinsic properties of the cell and not the properties of the specialized bipolar-amacrine cell synapses.

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Acetylcholine receptor-aggregating factor is similar to molecules concentrated at neuromuscular junctions

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The basal lamina in the synaptic cleft of the vertebrate skeletal neuromuscular junction contains molecules that direct the formation of synaptic specializations in regenerating axons and muscle fibres¹⁻⁴. We have undertaken a series of experiments aimed at identifying and characterizing the molecules responsible for the formation of one of these specializations, the aggregates of acetylcholine receptors (AChRs) in the muscle fibre plasma membrane. We began by preparing an insoluble, basal lamina-containing fraction from *Torpedo californica* electric organ, a tissue which has a far higher concentration of cholinergic synapses than muscle, and showing that this fraction caused AChRs on cultured chick myotubes to aggregate⁵⁻⁷. A critical step is learning whether or not the electric organ factor is similar to the receptor-aggregating molecule in the basal lamina at the neuromuscular junction. The importance of this problem is emphasized by reports that clearly non-physiological agents, such as positively charged latex beads⁸, can cause AChR aggregation on cultured muscle cells. We have already shown that *Torpedo* muscle contains an AChR-aggregating factor similar to that of electric organ, although in much lower amounts⁶. Here we demonstrate, using monoclonal antibodies, that the AChR-aggregating factor in our extracts of electric organ is, in fact, antigenically related to molecules concentrated in the synaptic cleft at the neuromuscular junction.

A mouse was injected with 300 μ g of an electric organ extract enriched $> 1,000$ -fold in AChR-aggregating activity⁷ and hybridomas were generated by standard procedures (see Table 1 legend). To identify hybridomas that produced antibodies against electric organ material, we used indirect immunofluorescence histochemistry and screened for antibodies that bound to formaldehyde-fixed frozen sections of electric organ. We then tested the antibodies that stained electric organ for those that could immunoprecipitate the AChR-aggregating activity from our electric organ extract. Thus far, we have cloned two

hybridomas producing such antibodies. Both antibodies, 6D4 and 2F6, also immunoprecipitated the activity in muscle extracts and blocked AChR aggregation when added to myotube cultures together with either muscle or electric organ extract (Table 1).

To determine the distribution of muscle antigens recognized by 6D4 and 2F6, formaldehyde-fixed frozen sections of *Torpedo* skeletal muscle were examined by immunofluorescence histochemistry. Figure 1 shows that 6D4 intensely stained the neuromuscular junctions. Staining was also detected in the walls of arteries and around individual pre-terminal axons, and there were occasional lightly stained patches at the extrajunctional surface of some muscle fibres (not shown). No other structures in muscle were labelled. Thus, the AChR-aggregating factors in our extracts of electric organ and muscle are antigenically related to molecules associated with more than one tissue component of muscle, but on myofibres, such molecules are concentrated at neuromuscular junctions.

Sections of muscle labelled with 2F6 had a staining pattern similar to that of 6D4, suggesting that both monoclonal antibodies bind to the same antigens. However, 2F6 did not stain sections exposed to fixative for longer than 1 min, even at a 100-fold greater concentration than that sufficient to stain unfixed tissue. Binding of 6D4, on the other hand, was not affected by our routine fixation; the titre required for staining was the same for unfixed sections and sections fixed for 5 min. Thus, 6D4 and 2F6 recognize epitopes which can be distinguished from one another by their sensitivity to fixation.

We are now performing experiments to determine whether antigens recognized by 6D4 and 2F6 at the neuromuscular junction are components of the synaptic basal lamina. As a first step, we checked the distribution of basal lamina in *Torpedo* muscle by staining cross-sections with an antiserum⁹ against laminin, a common constituent of basal lamina sheaths. As expected, basal lamina envelops the same structures in *Torpedo*

muscle as in mammalian muscle¹⁰, including muscle fibres, arteries, capillaries, veins, perineurium and Schwann cells (which surround axons). Thus, 6D4 and 2F6 do not stain all structures that have basal lamina, but basal lamina is associated with all structures stained by these antibodies. To determine whether or not epitopes recognized by 6D4 are extracellular, muscles were fixed, incubated with the antibody and examined in whole mounts by immunohistochemistry. The plasma membranes remained intact during these procedures; thus, the antibodies could interact only with the extracellular matrix and the external surface of plasma membranes. Fluorescence microscopy revealed that all the structures labelled in cross-sections were also stained in the whole mounts, including neuromuscular junctions (Fig. 1c). Some whole-muscle preparations incubated with 6D4 and a horseradish peroxidase (HRP)-conjugated second antibody were examined by electron microscopy to determine the distribution of 6D4 epitopes at neuromuscular junctions. Figure 1d demonstrates that HRP reaction product occupied much of the synaptic cleft, including the synaptic portion of the myofibre basal lamina; no labelling was detected beyond the synaptic cleft. Thus, epitopes recognized by 6D4 at neuromuscular junctions are concentrated in the synaptic cleft and are a part of or adjacent to the synaptic basal lamina.

Extracts that cause AChR aggregation on cultured myotubes have been obtained by others from neurone cultures¹¹ and neurone-rich tissues including brain, spinal cord and peripheral nerves¹²⁻¹⁷. None of the active factors in these extracts has been shown to be related to molecules concentrated at the neuromuscular junction or any other synapse, as we demonstrate here for the AChR-aggregating factor from *Torpedo* electric organ.

It is possible that the electric organ AChR-aggregating factor is related to a known component of the basal lamina at the neuromuscular junction. Such components include acetylcholinesterase¹⁸, a heparan sulphate proteoglycan¹⁹, and one or more uncharacterized antigens²⁰, all of which are either absent from, or in much lower concentrations in, extrajunctional regions of the myofibre basal lamina sheaths. The ability of these molecules to cause receptor aggregation has not been tested. Other components of synaptic basal lamina, including type IV collagen, fibronectin and laminin, are common to synaptic and extrasynaptic basal lamina¹⁰. Of these shared components, only laminin causes AChR aggregation when applied to cultured myotubes²¹. As reported previously, gel filtration of our solubilized electric organ material did not reveal any AChR-aggregating activity in the size range of laminin (relative molecular mass 880,000)⁹; most of the activity eluted at a position characteristic of proteins of lower relative molecular mass (50,000-100,000)⁷. Moreover, as illustrated in Table 2, antiserum against laminin, which stained basal lamina in our sections of *Torpedo* muscle, did not immunoprecipitate AChR-aggregating activity from electric organ extracts. These observations, coupled

Table 1 Monoclonal antibodies block and immunoprecipitate AChR-aggregating activity in extracts of electric organ and muscle

Additions	Activity (%)	
	Immunoprecipitation	Blocking
Electric organ extract		
+control	100 ± 12	100 ± 13
+6D4	5 ± 2	2 ± 2
+2F6	23 ± 5	9 ± 0
Muscle extract		
+control	100 ± 7	100 ± 29
+6D4	3 ± 4	31 ± 12
+2F6	3 ± 0	18 ± 8

A pH 5.5 citrate extract⁶ containing 1-1.5 units of AChR-aggregating activity prepared from *Torpedo* electric organ or tail muscle was mixed with 120-200 µl of hybridoma supernatant for 2 h at 37 °C in a volume of 200-465 µl. To determine blocking, aliquots were applied directly to cultured chick myotubes. To determine immunoprecipitation, the mixture was incubated for an additional 0.5-2 h with goat anti-mouse IgG-conjugated Sepharose beads (see ref. 6), centrifuged to remove the bound immune complexes, and aliquots of the supernatant were assayed on cultured myotubes. AChR-aggregating activity was determined by counting the number of patches of AChRs on the myotubes as described previously⁶. Control supernatants were taken from either the parent myeloma or a hybridoma producing antibodies that did not stain electric organ. Each value represents the mean ± s.e.m. of triplicate determinations. Generation of monoclonal antibodies: A female BALB/c mouse was given an intraperitoneal (i.p.) injection of 20 µg of purified electric organ extract⁷ emulsified with an equal volume of Freund's complete adjuvant; after 4 weeks, the mouse was injected i.p. with an additional 16 µg of this extract emulsified with Freund's incomplete adjuvant. After 4 more weeks, 260 µg of extract without adjuvant was injected beneath the capsule of the spleen. The spleen was removed 3 days later and the cells were fused with a mouse myeloma cell line, P3X63-Ag8.653, by the procedure of Oi and Herzenberg²³. Viable hybridomas were found in 500 of the original 572 wells plated. Of these, the supernatant from 105 wells stained sections of electric organ.

Table 2 Antiserum against laminin does not immunoprecipitate AChR-aggregating activity

Additions	Activity (%)
None	100 ± 16
Normal serum (20 µl)	122 ± 13
Antiserum against pH 5.5 citrate extract (2 µl)	5 ± 12
Antiserum against laminin (20 µl)	104 ± 13

An aliquot of a pH 5.5 citrate extract⁶ containing 1.5 units of AChR-aggregating activity prepared from *Torpedo* electric organ was mixed with the indicated amount of heat-inactivated rabbit serum in a total volume of 70 µl for 2 h at 37 °C. The mixture was incubated for an additional 0.5 h with 10 µg of fixed *Staphylococcus aureus* (Pansorbin [Calbiochem]), washed and resuspended in 50 µl of 1 M Tris buffer, pH 8.5, then centrifuged to remove bound immune complexes and assayed as described in Table 1 legend. Antiserum against pH 5.5 citrate extract⁶ was used to confirm the effectiveness of *S. aureus* immunoprecipitation.

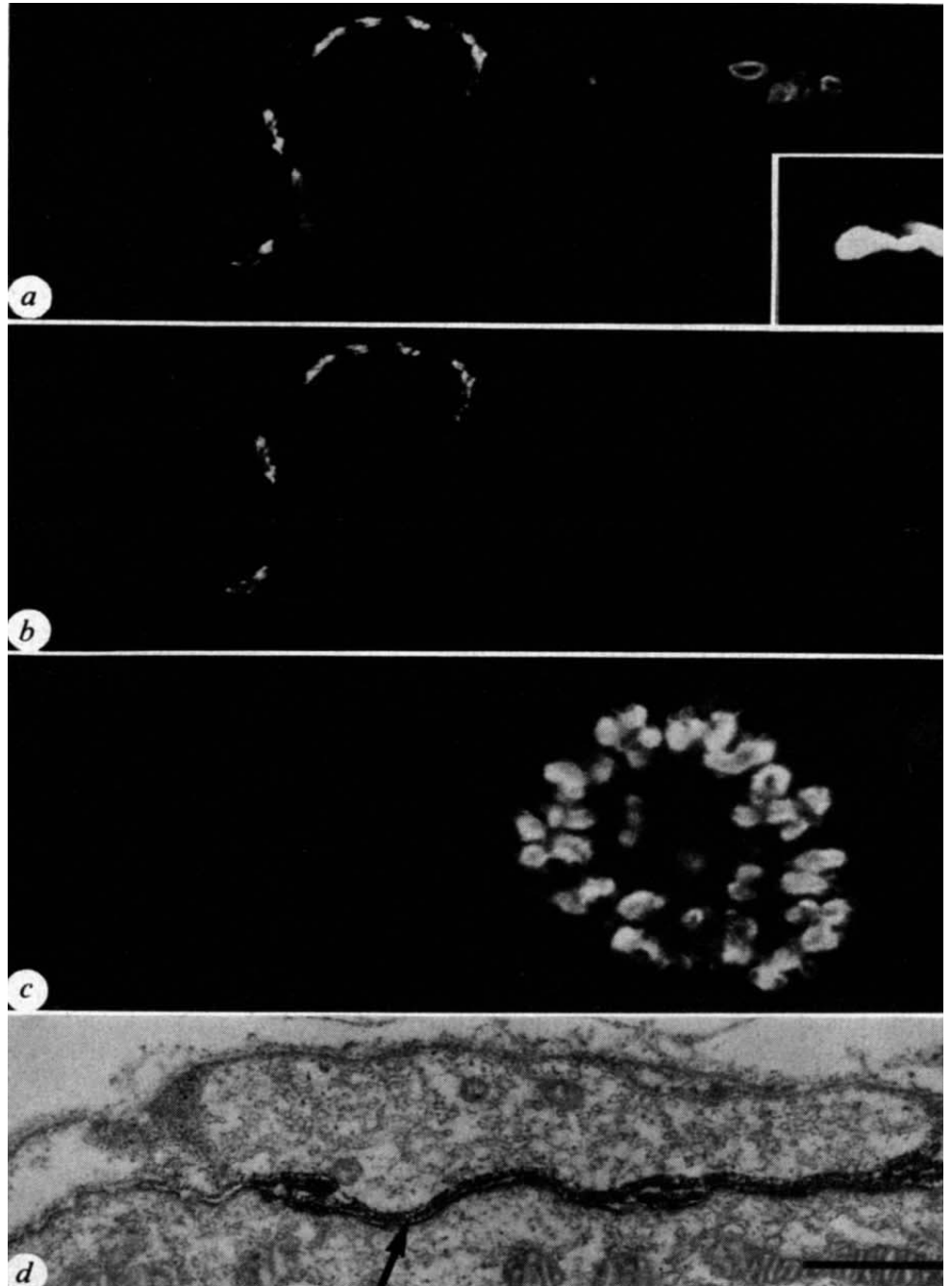
with the finding that 6D4 and 2F6 staining co-localized with only a fraction of anti-laminin staining in muscle, indicate that the electric organ AChR-aggregating factor is not laminin. However, the fact that two different monoclonal antibodies against the electric organ AChR-aggregating factor recognize cell-surface antigens in the walls of arteries and in nerves as well as at neuromuscular junctions may mean that the factor is

structurally related to one or a family of basal lamina molecules that have a broad distribution in muscle.

Taken together, our studies provide several lines of evidence that the electric organ AChR-aggregating factor is similar to the component of the synaptic basal lamina that directs the accumulation of AChRs on regenerating muscle fibres: the factor is found in an extracellular matrix-rich fraction of the electric

Fig. 1 6D4 binds to antigens concentrated at neuromuscular junctions in skeletal muscle. **a**, Frozen section of *Torpedo californica* muscle viewed with fluorescein optics to show the position of 6D4-binding sites. **b**, Same field as in **a** but viewed with rhodamine optics to reveal the location of neuromuscular junctions. 6D4 binding is confined to neuromuscular junctions, the wall of a collapsed artery (inset), and the perimeter of each of several axons. **c**, A whole-mount of a fascicle of *Torpedo* muscle fibres viewed with fluorescein optics to reveal bound 6D4. A neuromuscular junction, seen from above and similar in layout to that of other vertebrates²⁴, is intensely stained; pre-terminal axons are faintly stained. **d**, Electron micrograph of a neuromuscular junction from a *Discopyge ommata* fin muscle incubated with 6D4 and then with an HRP-conjugated second antibody. Electron-dense HRP reaction product occupies much of the synaptic cleft, including the synaptic portion of the muscle fibre basal lamina (arrow). We used the fin muscles from *D. ommata* (an electric ray like the *Torpedo* but less than one-quarter the size) because they were smaller than any muscles we encountered in *Torpedo* and, thus, were easier to process for electron microscopy. Fluorescence immunohistochemistry on frozen sections and on whole mounts of these muscles revealed the same 6D4 staining pattern as in muscles from *Torpedo*; no 6D4 staining was observed at neuromuscular junctions in frog, salamander and chick muscles. Scale bar: **a**, **b**, 75 μ m; **c**, 33 μ m; **d**, 1 μ m.

Methods. Frozen sections **a**, **b**: sections (8–10 μ m thick) of back muscles of *Torpedo* were incubated for 10 min in 10% normal goat serum in phosphate-buffered saline (PBS), washed briefly in PBS, fixed for 5 min in 1% formaldehyde in PBS, washed for 5 min in 0.1% Triton X-100 in PBS (PBS-T), and incubated overnight at 4 °C in 6D4 supernatant diluted 1:200 in PBS containing 20% normal goat serum and 0.5% Triton X-100 (PBS-ST). (The same staining pattern was observed at dilutions of 6D4 supernatant ranging over 1–20,000-fold.) The sections were then washed for 3 h in PBS-T and incubated for 1 h in PBS-ST containing fluorescein-conjugated goat anti-mouse IgG (Cappel) to label bound 6D4 and rhodamine- α -bungarotoxin to stain AChR aggregates at neuromuscular junctions. Coverslips were mounted with Citifluor mountant medium AF1 (City University, London). Whole mounts (**c**): Abdominal muscles bathed in *Torpedo* Ringer's²⁵ were dissected down to single fascicles three to five muscle fibres thick. The muscles were then fixed for 1 h with 1% paraformaldehyde in Ringer's solution, incubated overnight at 4 °C in 6D4 supernatant diluted 1:200 with 0.3 M sodium phosphate buffer pH 7.1 (PB), washed for 8 h with several changes of PB, incubated overnight in fluorescein-conjugated goat anti-mouse IgG (Cappel) diluted 1:800 with PB, washed for 3 h in phosphate buffer with 0.1% Triton X-100 (PB-T), refixed for 0.5 h in 1% paraformaldehyde, washed for 2 h with PB-T, and mounted with mountant medium AF1 between glass coverslips. Electron microscopy (**d**): Muscles were pinned out in Ringer's solution, incubated for 3 h in 6D4 diluted 1:50 with Ringer's solution, washed for 45 min with Ringer's, incubated in Ringer's containing HRP-conjugated goat anti-mouse IgG (Cappel), washed for 45 min in PB, fixed for 30 min in 1% glutaraldehyde in PB, washed for 30 min with 0.25 M cacodylate buffer pH 7.1, incubated for 30 min in 0.05% 3,3'-diaminobenzidine (Sigma) and 0.02% H₂O₂ in cacodylate buffer, washed for 30 min in cacodylate buffer, immersed for 1 h in 1% osmium tetroxide in PB, dehydrated with ethanol and embedded in a mixture of Epon and Araldite³.



organ⁶; the corresponding fraction of *Torpedo* muscle contains a similar activity⁶; antiserum against partially purified electric organ factor blocks and immunoprecipitates AChR-aggregating activity from muscle and stains muscle basal lamina⁷; as demonstrated here, two monoclonal antibodies recognizing different epitopes block and immunoprecipitate the active component in extracts of both electric organ and muscle and bind in high concentration at the neuromuscular junction; and, at least one of the monoclonal antibodies recognizes molecules that are in or adjacent to synaptic basal lamina. Thus, monoclonal antibodies against the electric organ AChR-aggregating factor, such as 6D4 and 2F6, may be useful reagents for identifying and purifying the components of synaptic basal lamina that direct the aggregation of AChRs on regenerating myofibres, for studying their mechanism of action, for learning how they are regulated and for determining whether they also direct the formation of receptor aggregates in developing muscles or the maintenance of such aggregates in normal adult muscles. It is now known that synaptic basal lamina directs the aggregation of acetylcholinesterase as well as that of AChRs in regenerating muscle⁴. As described in the following report²², 6D4 and 2F6 have been used to provide evidence that the electric organ factor which causes AChR aggregation also causes the accumulation of acetylcholinesterase on the surface of cultured myofibres, raising the possibility that a single molecule in the basal lamina directs the differentiation of both of these postsynaptic specializations in regenerating muscle.

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Aggregates of acetylcholinesterase induced by acetylcholine receptor-aggregating factor

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Basal lamina-rich extracts of *Torpedo californica* electric organ contain a factor that causes acetylcholine receptors (AChRs) on cultured myotubes to aggregate into patches¹⁻³. Our previous studies have indicated that the active component of these extracts is similar to the molecules in the basal lamina which direct the aggregation of AChRs in the muscle fibre plasma membrane at regenerating neuromuscular junctions *in vivo*^{2,4,5}. Because it can be obtained in large amounts and assayed in controlled conditions in cell culture, the AChR-aggregating factor from electric organ may be especially useful for examining in detail how the postsynaptic apparatus of regenerating muscle is assembled. Here we demonstrate that the electric organ factor causes not only the formation of AChR aggregates on cultured myotubes, but also the formation of patches of acetylcholinesterase (AChE). This finding, together with the observation that basal lamina directs the formation of both AChR and AChE aggregates at regenerating neuromuscular junctions *in vivo*⁶, leads us to hypothesize that a single component of the synaptic basal lamina causes the formation of both these synaptic specializations on regenerating myofibres.

First, we investigated whether extracts of *Torpedo* electric organ would affect the distribution of AChE on myotubes. For this, aliquots of a saline- and detergent-insoluble fraction of electric organ³ were added to 5-day-old chick myotube cultures; this fraction is rich in components of basal lamina and causes AChRs on cultured myotubes to aggregate³. After 24 h, treated and control cultures were fixed and stained for cholinesterase

Table 1 Histochemically stained patches contain myotube-derived ChE

	Addition	Aggregates per myotube segment
Expt a		
	Control	None
	Eserine	1.1 ± 0.1
	Insoluble fraction	0.5 ± 0.2
Expt b		
	Control	None
	MSF-treated medium	8.9 ± 0.1
	Insoluble fraction	0.9 ± 0.3
Expt c		
	Control	None
	MSF-treated medium	2.1 ± 0.2
	Insoluble fraction	2.1 ± 0.5
Expt d		
	Control	None
	MSF-treated medium	4.2 ± 0.3
	Insoluble fraction	4.5 ± 1.2

Expt a, 40-μl aliquots of a MSF-treated insoluble fraction (6.9 μg) or MSF-treated medium were added to cultures (see Fig. 1 legend for MSF treatment). The cultures were incubated overnight and stained for cholinesterase in the presence or absence of 10⁻⁵ M eserine. Eserine was added 10 min before the histochemical reaction was begun and was present throughout the 1½-h incubation. Expt b, MSF (final concentration, 2 mM) was added to culture medium and the mixture was incubated at room temperature for 1 h. Aliquots of the insoluble fraction, treated with MSF as in a, were then added to control cultures and cultures that had been fed with MSF-treated medium. The cultures were incubated overnight and stained for cholinesterase. Data are expressed as mean ± s.e.m. of triplicate determinations.

(ChE) activity by the histochemical method of Karnovsky⁷. Figure 1a, b shows that the insoluble fraction caused a marked increase in the number of patches of reaction product on the myotubes. Few patches were seen on myotubes stained in the presence 10⁻⁵ M eserine (Table 1), indicating that the reaction product was due to cholinesterase activity. Patches were induced when both the culture medium and the insoluble fraction were pretreated with methane sulphonyl fluoride (MSF), an irreversible inhibitor of ChE⁸ (Table 1); therefore, the ChE in the patches must have been produced by the myotubes. To determine

Fig. 1 Formation of AChE aggregates by *Torpedo* electric organ extracts. **a, b**, Bright-field photographs of 5-day chick myotube cultures³, incubated for 24 h with medium containing 16 μ g of an insoluble fraction of electric organ (**a**) or normal medium (**b**) and stained for ChE activity. **c**, Fluorescence micrograph of a 5-day culture exposed for 24 h to 45 ng of agarose pool and labelled with anti-AChE and fluorescent second antibody. Scale bars, 50 μ m.

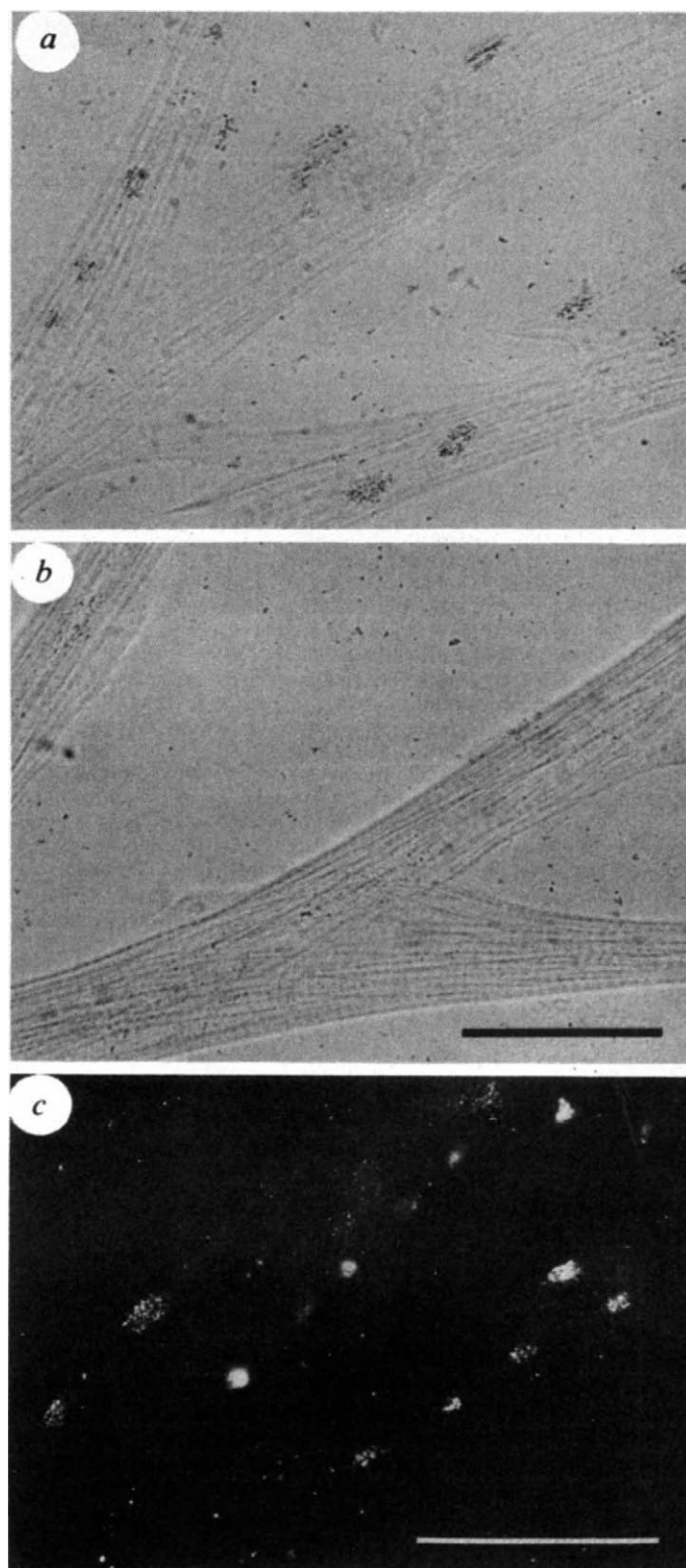
Methods. **a, b**, The cultures were rinsed with buffer containing 150 mM NaCl, 10 mM sodium phosphate, pH 7.4 (PBS), fixed for 5 min in 1% paraformaldehyde in 90 mM sodium phosphate, 130 mM sucrose, pH 7.4, rinsed with PBS and then reacted for cholinesterase by the method of Karnovsky⁷ for 1½ h at room temperature. Cultures were rinsed with 0.1 M sodium maleate, pH 6.0, with water, then dehydrated in graded ethanols and mounted in glycerol. Methane sulphonyl fluoride (final concentration 2 mM) was added to the insoluble fraction and the mixture incubated for 1 h at room temperature before adding it to the cultures. The MSF treatment inhibited cholinesterase in the insoluble fraction by >99.8%, as assayed by the method of Ellman *et al.*²⁶. The rapid spontaneous hydrolysis of MSF prevented it from inhibiting ChE on the myotubes. **c**, AChE was labelled with a monoclonal antibody to chick AChE and fluorescent second antibody as follows. Cultures were rinsed with PBS, fixed for 30 min in 1% paraformaldehyde in 90 mM sodium phosphate, 130 mM sucrose, pH 7.2, rinsed with PBS and incubated for 1 h at room temperature with a mouse monoclonal antibody specific for chick AChE, 1A2 anti-AChE⁹ (6 μ g ml⁻¹ in PBS supplemented with 10% horse serum). The cultures were then rinsed with PBS, incubated for 1 h with a 1/200 dilution of rhodamine-conjugated goat anti-mouse IgG (Cappel) in PBS + 10% horse serum, rinsed with PBS, dehydrated in graded ethanols and mounted in glycerol. No immunohistochemical staining was seen when cultures were incubated with antibodies secreted by the parent myeloma (not shown).

Table 2 Comparison of ChE histochemistry and AChE immunohistochemistry

	Aggregates per myotube segment	
	ChE histochemistry	Anti-AChE immunohistochemistry
Expt a		
Control	3.0 (2.6, 3.4)	3.3 (2.8, 3.8)
Insoluble fraction	6.4 (6.3, 6.6)	5.3 $\pm$ 0.6
Expt b		
Control	2.0 (1.8, 2.1)	4.8 $\pm$ 0.8
pH 5.5 extract	6.9 (6.2, 7.6)	10.5 $\pm$ 0.8
Expt c		
Control	1.6 $\pm$ 0.3	1.3 $\pm$ 0.2
Agarose pool	7.2 $\pm$ 0.7	5.7 $\pm$ 0.8

Cultures were treated with the insoluble fraction, pH 5.5 extract or agarose pool and either stained for ChE activity or labelled immunohistochemically to reveal AChE (see Fig. 1 legend). Labelled patches on the bottom surface of the myotubes were counted (see Fig. 2 legend). The numbers of patches on 10 myotube segments 400 μ m long examined at $\times 250$ magnification were averaged for each culture histochemically. As the patches of rhodamine-labelled anti-AChE were difficult to see at this magnification, antibody-labelled patches were counted at $\times 400$ magnification and the numbers of patches on 20 myotube segments were averaged and multiplied by 1.6. Expts a, b and c were done on different sets of cultures. Data are expressed as the mean of duplicate or triplicate determinations. For experiments done in duplicate, the individual values are given in parentheses; for experiments done in triplicate, the s.e.m. is shown.

whether the patches induced by the electric organ fraction contained true AChE, control cultures and cultures treated with the insoluble fraction were fixed and labelled with a monoclonal antibody specific for chick AChE⁹. The electric organ fraction caused an increase in the number of patches of antibody-labelled AChE on the surface of the myofibres similar to the increase seen with histochemical staining (Fig. 1c, Table 2). Because the labelling protocol used for these experiments did not allow antibodies to penetrate plasma membranes, the AChE patches detected must be on the myotube surface. Thus, the insoluble fraction of electric organ causes not only the aggregation of



AChRs on cultured myotubes, but also the formation of aggregates of AChE.

We next sought to establish a quantitative assay for AChE-aggregating activity, so that its level could be compared with that of the AChR-aggregating factor. Figure 2 shows that as the amount of the insoluble fraction added to cultures was increased, the number of AChE patches increased and then reached a plateau. Although the magnitude of the response to saturating amounts of the insoluble fraction varied over 2–9-fold from plating to plating, there was little variability in the dose depen-

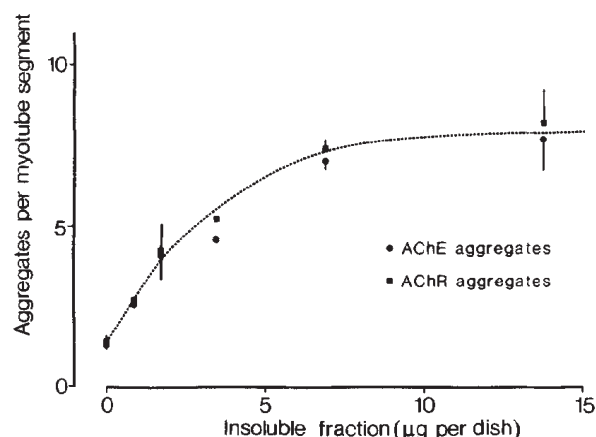


Fig. 2 Dose dependence of the formation of AChR (■) and AChE (●) aggregates on cultured myotubes. Cultures, treated for 24 h with the indicated amount of insoluble fraction, were incubated with 6×10^{-8} M rhodamine- α -bungarotoxin²⁷ in culture medium for 1 h at 37 °C. The cultures were then rinsed twice with PBS and fixed and reacted for cholinesterase activity as described in Fig. 1 legend. AChR and ChE clusters were counted at $\times 250$ magnification with a Nikon microscope equipped with phase and fluorescence optics. The ChE-stained patches and α -bungarotoxin-labelled AChR clusters were often seen to be partially or totally overlapping; however, there was no indication that the ChE staining ever completely obscured a toxin-labelled AChR aggregate. For each culture, myotubes were chosen at random and the numbers of patches of cholinesterase reaction product along unbranched 400- μ m-long segments of the myotubes were counted under phase optics; then the number of AChR clusters were counted under fluorescence optics. Only patches on the bottom of the myotubes were counted because most factor-induced clusters of AChRs and ChE occurred on this surface of the myotube. Counts from 10 myotubes, evenly spaced over the surface of the dish, were averaged. We counted as an aggregate any distinct fluorescent or cholinesterase-stained patch whose longest axis was $> 2 \mu$ m. Data are expressed as the mean $\pm$ s.e.m. ($n=3$); error bars are shown where they exceed the size of the data point. To quantitate AChR- and ChE-aggregating activity, curves were drawn through the points by eye (the two curves superimpose in this figure) and one unit of activity was taken as the amount of the fraction required to give a half-maximal response (in this case 2.58 μ g per dish).

dence of the response. Thus, by defining one unit of activity as the amount of material required to cause a half-maximal response, AChE-aggregation could be measured reproducibly between experiments.

The dose dependence of the insoluble fraction-induced formation of AChE patches on cultured myofibres was the same as for AChR aggregation (Fig. 2), suggesting that a single factor caused both effects. If this were the case, it should be possible to extract the AChE-aggregating activity from the insoluble fraction and purify it by procedures that solubilize and purify the AChR-aggregating factor. Accordingly, the insoluble fraction was extracted with pH 5.5 citrate buffer³, the solubilized material was fractionated by gel filtration chromatography on agarose² and the column fractions causing AChR aggregation were pooled. Both AChR- and AChE-aggregating activities were measured in the insoluble fraction, the pH 5.5 extract and the 'agarose pool'. Table 3 shows that the AChR- and AChE-aggregating specific activities were increased to the same extent by extraction and gel filtration chromatography and that the dose dependence for AChR aggregation was the same as that for the formation of AChE aggregates at each step in the purification. (Formation of AChE aggregates was routinely assayed by ChE histochemistry; however, immunohistochemical experiments confirmed that each fraction induced patches that contained true AChE [Table 2]). Thus, AChE- and AChR-aggregating activities co-purify. We also examined whether or not two different monoclonal antibodies, 6D4 and 2F6, directed against the AChR-aggregating factor (see accompanying report¹⁰), would recognize the AChE-aggregating factor (Table

Table 3 Co-purification of AChE- and AChR-aggregating activities

Fraction	Aggregating activity (units per mg protein)	
	AChR	AChE
Insoluble fraction	374	382
pH 5.5 extract	811	952
Agarose pool	49,800	56,200

For each fraction, AChE- and AChR-aggregating activities were measured as described in Fig. 2 legend. The values are the mean of two separate determinations.

Table 4 Immunoprecipitation (a) and inhibition (b) of AChE- and AChR-aggregating activities

	Addition	Aggregates per myotube segment (% control)	
		AChE	AChR
Expt a	Control	100 $\pm$ 16	100 $\pm$ 15
	6D4	-2 $\pm$ 2	-1 $\pm$ 4
	2F6	-1 $\pm$ 4	7 $\pm$ 3
Expt b	Control	100 $\pm$ 5	100 $\pm$ 5
	6D4	2 $\pm$ 11	11 $\pm$ 4
	2F6	22 $\pm$ 4	10 $\pm$ 3

Expt a: An aliquot of agarose pool (7.2-8.2 units, 134-150 ng) was mixed with 0.3-1.3 ml hybridoma supernatant and incubated for 1½ h at 37 °C. Immunoglobulins and immune complexes were precipitated by adding 30-50 μ l of a suspension of goat anti-mouse antibodies covalently coupled to Sepharose beads³ and 100 μ l aliquots of the supernatant were assayed for aggregating activity. Control supernatant was from a hybridoma secreting antibodies of unknown specificity. Expt b: An aliquot of agarose pool (6.5-8.2 units, 120-150 ng) was mixed with 0.35-1.3 ml myeloma-conditioned medium or hybridoma supernatant, incubated for 1 h at 37 °C and 0.12-0.4 ml aliquots were assayed for aggregating activity. Data for both experiments, expressed as mean $\pm$ s.e.m. ($n=3$), are per cent of maximal response.

4). When bound to Sepharose beads, 6D4 and 2F6 each removed AChR- and AChE-aggregating activity from the agarose pool. Moreover, when added directly to cultures together with the agarose pool, 6D4 and 2F6 inhibited the extract-induced formation of both AChR and AChE aggregates. From the observations that (1) the AChR- and AChE-aggregating activities co-purify, (2) the dose dependence for the induction of AChR and AChE aggregates is the same at each stage of the purification and (3) two different monoclonal antibodies immunoprecipitate and inhibit both activities, we conclude that a single factor in our extracts of electric organ causes the formation of AChR and AChE patches.

AChR and AChE aggregates form part of the postsynaptic apparatus at both normal and regenerated neuromuscular junctions and are the only components of the apparatus known to be directly involved in synaptic transmission. The formation of these cell-surface specializations at developing neuromuscular junctions is induced by the axon terminal¹¹⁻¹³. On the other hand, in adults, the synaptic portion of the myofibre's sheath of basal lamina can direct the aggregation of AChRs and AChE on regenerating myofibres⁴⁻⁶. Thus, the synaptic basal lamina in the adult contains synaptogenic information similar to that provided by the axon in the embryo.

The identity and mechanism of action of the molecules that induce the postsynaptic specializations at developing and regenerating neuromuscular junctions are unknown, although *in vivo* and *in vitro* studies indicate that electromechanical activity of muscle fibres is necessary for the accumulation of high levels of AChE¹⁴⁻¹⁶. Crude extracts from several neurone-rich tissues such as brain, spinal cord and nerve have been shown to cause AChR aggregation on cultured myotubes, and the active components of some have been partially purified¹⁷⁻²⁵. Furthermore, many of the AChR patches formed in response to at least one of the brain extracts are associated with synapse-specific anti-

gens, AChE and invaginations in the myotube plasma membrane which resemble the junctional folds found at normal and regenerating neuromuscular junctions²⁵. The results reported here extend the findings of such studies by showing that, in the basal lamina-containing fraction of electric organ, the same factor that causes AChR aggregation also causes the formation of aggregates of AChE. We have also demonstrated (see accompanying report¹⁰) that two different monoclonal antibodies against this AChR/AChE-aggregating factor bind in high concentration at the neuromuscular junction and that the molecules recognized by at least one of the monoclonal antibodies are in or adjacent to the synaptic basal lamina. Thus, it is reasonable to suggest that the molecules in the synaptic basal lamina which direct the formation of AChR aggregates on regenerating myofibres also cause aggregation of AChE. We are now performing experiments to test this monomolecular induction hypothesis for the formation of AChR and AChE aggregates at both regenerating and developing neuromuscular junctions and to discover whether the electric organ AChR/AChE-aggregating factor causes the formation of other components of the postsynaptic apparatus.

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Inhibition of the Prausnitz-Küstner reaction by an immunoglobulin ϵ -chain fragment synthesized in *E. coli*

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The Prausnitz-Küstner (P-K) reaction is a sensitive test for the presence and activity in the skin of immunoglobulin E, an important class of immunoglobulin mediating allergic reactions. A fragment of the human myeloma ND ϵ -chain gene, encoding the second, third and fourth domains of the IgE constant region (C ϵ 2-4), was assessed here for its ability to inhibit the P-K reaction *in vivo*. Injection of the fragment in skin sites of healthy human adults prevented subsequent sensitization with serum containing IgE anti-

body to ragweed antigen. Inhibition of the P-K reaction required a 200-fold molar excess of the C ϵ fragment over the IgE present in the sensitizing serum. The efficacy of the C ϵ fragment in inhibiting the P-K reaction compared favourably with that of natural myeloma IgE (PS) in terms of both blocking concentrations and duration of the blocking effect. The inhibition of the P-K reaction by C ϵ 2-4 fragments was specific and probably caused by the saturation of IgE receptors on mast cells^{1,2} by the recombinant gene product.

The C ϵ 2-4 gene fragment used encodes amino acids 218-547 of the IgE molecule and was subcloned in *Escherichia coli*, producing a polypeptide of relative molecular mass 40,000³. Preparations consisting of >95% affinity-purified C ϵ 2-4 dimeric chain fragments, when injected into skin sites of a human volunteer, completely blocked the passive sensitization of these sites by serum (E.C.) containing IgE antibody to ragweed antigen (Table 1). This blockade was prevented by treating the C ϵ 2-4 fragments at 56 °C for 1 h or by reduction and alkylation. Skin sites injected with C ϵ 2-4 fragments remained normally responsive to histamine (data not shown). More importantly, skin sites sensitized with reaginic serum (E.C.) 1 h before C ϵ 2-4 fragment injection retained their full ability to support a P-K reaction.

The P-K reaction under study was caused by IgE antibody to ragweed antigen in the sensitizing serum. Heat treatment (56 °C, 1 h) of serum E.C. abolished its ability to sensitize normal skin (data not shown), as would be the case for an IgE antibody^{4,5}. Table 2 shows that when an immunoglobulin-enriched fraction of serum E.C. was fractionated over an anti-IgE (PS) Sepharose-4B column, only the IgE-containing eluate, but not the IgE-depleted effluent, was able to induce a P-K reaction. This was blocked by pretreatment of the skin sites with C ϵ 2-4 fragments.

The P-K reaction induced by a 1:100 dilution of serum E.C. (1:100) containing 5×10^{-11} M IgE was abolished completely

Table 1 Inhibition of Prausnitz-Küstner reaction by C ϵ 2-4 fragments

	Inhibitor	Area (mm ²) of wheal and flare at sites sensitized with	
		Serum E.C. diluted 1:10	Serum E.C. diluted 1:100
Expt 1	Diluent	87/480	42/257
	C ϵ 2-4 (100 μ g ml ⁻¹)	0/0	0/0
	C ϵ 2-4 56 °C 1 h (100 μ g ml ⁻¹)	76/455	38/271
Expt 2	Diluent	103/516	54/268
	C ϵ 2-4 (10 μ g ml ⁻¹)	0/0	0/0
	C ϵ 2-4 (10 μ g ml ⁻¹) reduced, alkylated	92/472	44/240

Values are the areas of the wheal/the area of the flare. Reduction and alkylation were performed as described in ref. 3. The same subject was used for passive sensitization in both experiments; the serum IgE of this subject was 4 IU ml⁻¹ (~10 ng ml⁻¹). The sensitizing serum (E.C.) contained 380 IU ml⁻¹ of IgE (~912 ng ml⁻¹), of which 8.7% was directed against ragweed antigen, as determined by the specific drop in serum IgE following absorption of the serum over a Sepharose 4B ragweed antigen column compared with a control Sepharose 4B human serum albumin⁷. Serum E.C. was free of detectable hepatitis B antigen and of antibodies to that antigen. C ϵ 2-4 fragments were injected into skin sites 1 h before the injection of serum E.C. Skin sites were challenged 48 h later with ragweed antigen (1,000 protein nitrogen units ml⁻¹ of a mixture of giant and short ragweed; Hollister-Stier). Twenty minutes later the skin sites were examined for the presence of wheal and erythema. The surface area of the reactions was estimated as follows. Transparent tape was used to transfer the outline of the reactions to paper⁸ which was cut and weighed on an analytical balance. The area was read from a standard curve. Diluent consisted of 0.15 M NaCl and 0.03% human serum albumin. All injections were intradermal and 0.02 ml in volume. In each experiment a set of skin sites were also sensitized with diluent. None of these sites showed a wheal or flare reaction when challenged with ragweed antigen.

by concentrations of C_e2-4 fragments as low as 1×10^{-8} M and concentrations of IgE (P.S.) as low as 1×10^{-9} M. Thus, a 200-fold excess of C_e2-4 and a 20-fold excess of IgE (PS) over IgE present in serum E.C. was required to block the P-K reaction. The latter result agrees well with the results of Stanworth *et al.*⁶, who found that a 10-100-folds excess of myeloma IgE (ND) over reaginic serum IgE was required to block the P-K reaction. When serum E.C. was diluted to 5×10^{-12} M IgE, it lost its ability to produce a P-K reaction. As 1×10^{-8} M C_e2-4 completely inhibited the ability of 5×10^{-11} M IgE in E.C. serum to cause a P-K reaction, it is likely that the 200-fold excess of C_e2-4 inhibited serum IgE to skin mast cells *in vivo* by 90%. *In vitro*, a 100-fold molar excess of C_e2-4 fragments inhibited 125 I-IgE PS binding by 80% (ref. 3). Thus, the ability of C_e2-4 fragments to inhibit IgE binding to mast cells *in vivo* and *in vitro* is comparable.

Comparison of the relative amounts of C_e2-4 fragments and IgE (PS) needed to produce 50% reduction of the area of skin erythema induced by the P-K reaction shows that the inhibitory activity of the C_e2-4 fragments was, on a molar basis, 30% that of natural IgE (Fig. 1). This is close to the value of 35% obtained

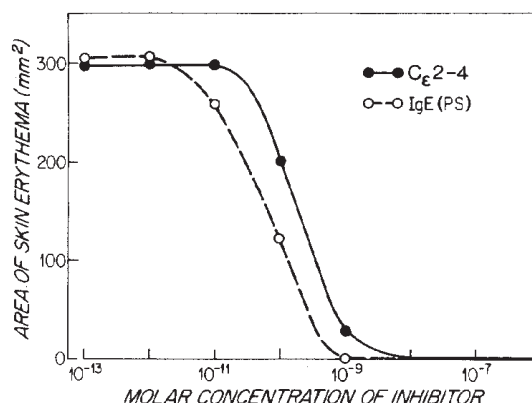


Fig. 1 Dose-response curve of inhibition of the Prausnitz-Küstner reaction. Skin sites in the forearm of a normal donor, whose serum IgE was <4 IU ml^{-1} , were injected 1 h before sensitization with decreasing concentrations of C_e2-4 (—), IgE (PS) (---) or diluent (Δ). A 1:100 dilution of the reaginic serum E.C. containing 5×10^{-11} M IgE was used to sensitize the sites which were challenged 48 h later with ragweed antigen as described in Table 1 legend. The size of the skin erythema (flare) is shown.

Table 2 Induction of the Prausnitz-Küstner reaction by IgE-enriched fractions of reaginic serum and its inhibition by C_e2-4 fragments

Serum (E.C.) fraction	IgE content (ng ml^{-1})	Area (mm^2) of wheal/flare at skin sites preinjected with diluent
Serum (1:100)	10	34/312
Ig enriched	7	22/210
IgE depleted	≤ 0.2	0/0
IgE enriched	7	27/238

An immunoglobulin-enriched fraction of serum E.C. was obtained by precipitation with 50% $(\text{NH}_4)_2\text{SO}_4$. The precipitate was reconstituted with 0.15 M saline to the initial serum volume, extensively dialysed against 0.15 M saline and diluted 1:100 in standard diluent before testing. An aliquot of the immunoglobulin-enriched fraction was circulated for 18 h at 4°C over a Sepharose anti-IgE (PS) column⁷. The anti-IgE (PS) was raised in a goat and rendered IgE specific by absorption with human IgM, IgA, IgG myelomas and κ , λ Bence-Jones proteins coupled to Sepharose. This was followed by absorption and elution on an IgE (DAZ) column. The effluent IgE-depleted fraction was absorbed twice more over other columns of anti-IgE (PS) Sepharose. IgE was eluted from the first absorbing column with glycine-HCl buffer, pH 3. Eluate and effluent were dialysed extensively against phosphate-buffered saline (PBS), then diluted in standard diluent and sterilized by Millipore filtration before use. The IgE content was assayed by a solid radioimmunoassay using monoclonal mouse anti-human IgE (gift of Dr R. Siraganian) to coat the plastic polyvinyl plates and 125 I-anti IgE ND (Pharmacia) as a developing reagent. The assay was carried as described in ref. 9. The sensitivity of the assay was 200 pg ml^{-1} . The recipient of the P-K reaction had a serum IgE of 50 IU ml^{-1} ($\sim 120 \text{ ng ml}^{-1}$).

Table 3 Duration of inhibition of the Prausnitz-Küstner reaction

Inhibitor	Inhibitor concentration 10^{-7} M	10^{-6} M
C_e2-4	12	17
IgE (PS)	14	21

Values given are days elapsed following injection of inhibitor before a successful P-K reaction could be achieved. Multiple skin sites of a normal donor with a serum IgE of 50 IU ml^{-1} were injected at day 0 with C_e2-4 fragments, IgE (PS) or diluent. At intervals of ≥ 2 days (days 0, 4, 9, 12, 14, 17, 19, 21), individual skin sites were sensitized with serum E.C. (1:100 dilution), then challenged 48 h later with ragweed antigen. Sites pretreated with diluent always gave a positive wheal and flare reaction with a mean $\pm$ s.d. area of the wheal of $56 \pm 9 \text{ mm}^2$ and a mean $\pm$ s.d. area of the flare of $400 \pm \text{mm}^2$ for the eight successive determinations. The days shown represent the time of the first appearance of any flare and/or erythema at the challenged skin sites.

for the relative efficacy of native IgE fragments in inhibiting 125 I-IgE binding to basophils *in vitro*³.

Table 3 shows that inhibition of the P-K reaction lasted for 12 days at sites that had received C_e2-4 fragments, compared with 14 days at sites that had received IgE PS, when each was used in a 2,000-fold molar excess over IgE present in serum E.C. This inhibition lasted 17 and 21 days, respectively, at sites that had received a 20,000-fold molar excess of C_e2-4 fragments or IgE PS.

The present experiments demonstrate that, in man, the P-K reaction can be blocked by a bacterial expression product of a human C_e2-4 gene fragment. The inhibitory efficacy of C_e2-4 fragments compared favourably with that of myeloma IgE (PS) both in terms of concentrations needed to inhibit the P-K reaction and in the duration of their blocking effect. As C_e2-4 fragments are not glycosylated, glycosylation of the IgE molecule may not be essential for mast cells binding *in vivo*.

The C_e fragment used in the present study contained the tryptophan control region of the expression plasmid pWT211 (ref. 3). Smaller C_e fragments lacking plasmid-derived peptides are currently being constructed and tested for inhibition of the P-K reaction. Because there are no known allotypic markers for C_e , such fragments should not be immunogenic to man. Lack of immunogenicity, potential availability in unlimited quantities and capacity to inhibit the P-K reaction for a relatively long period should make such fragments useful in attempts to prevent allergic reactions.

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Increased MHC *H-2K* gene transcription in cultured mouse embryo cells after adenovirus infection

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The class I major histocompatibility complex (MHC) antigens are highly polymorphic¹ cell-surface proteins² whose expression is essential for the cellular immune response against virus-infected, abnormal and foreign cells^{3,4}. Transformation of primary rat cell cultures by the oncogenic adenovirus 12 (Ad12) results in suppression of the transplantation antigens⁵, thus enabling the transformed cells to escape the immune response and efficiently form tumours *in vivo*⁶. In contrast, transformation of the same cells with the non-oncogenic adenovirus 5 (Ad5) does not suppress the transplantation antigens⁵ and, consequently, they elicit an effective (MHC-restricted) immune response⁶. Here, however, we show that infection of mouse embryo cells with both viruses initially increases the level of transcripts from the *H-2K^b* transplantation antigen gene. Both the adenovirus E1a (12S RNA) and E1b genes are required for activation of the *H-2K* gene and measurement of the relative rate of transcription indicates that the increase in the level of *H-2K* messenger RNA following infection is at least in part due to a gene-specific transcriptional activation. The newly transcribed *H-2K^b* mRNA is then properly transported to the cytoplasm.

Cell cultures from 16-day-old C57BL/10 mouse embryos were infected after second or third passage with wild type or mutants of Ad5. RNA was extracted 50 h after infection and the level of transcripts from the *H-2K^b* gene measured by *S₁* nuclease protection analysis using a 600-nucleotide *Ava*II probe which spans the exon III-intron III boundary of the *H-2K^b* gene⁷. As indicated by the relative intensities of the 230-nucleotide *S₁* nuclease-protected fragment (Fig. 1a), infection with Ad5 resulted in a 10–15-fold increase in the level of *H-2K^b* mRNA (lane 2) compared with mock-infected cells (lane 1). In contrast, infection with either the Ad5 *dl312* E1a deletion mutant or the Ad5 *dl313* E1b deletion mutant^{8,9} did not effect an increase in the level of *H-2K* mRNA (Fig. 1a, lanes 3, 4). To determine whether Ad5 infection caused a nonspecific change in the rate of gene transcription and/or RNA stability, we also measured the level of histone H4 gene transcripts in the above experiments. The level of histone H4 RNA increased only 2–3-fold after Ad5 infection under conditions in which the level of *H-2K^b* gene transcripts increased 15-fold (Fig. 1b).

Measurement of the relative rate of transcription of *H-2K^b* in nuclei isolated from mock-infected and Ad5-infected cells indicated that the increased level of *H-2K* mRNA following Ad5 infection was at least in part due to transcriptional activation. The activation appeared to be specific for *H-2K* as the rate of transcription of the type IV collagen gene did not increase on Ad5 infection (Fig. 1c).

Infection of mouse embryo cultures with Ad12 also resulted in a 10–15-fold increase in the level of total cellular *H-2K^b* mRNA (Fig. 2a, lane 1) compared with mock-infected cells (lane 9). Infection with a non-tumorigenic Ad12 E1a mutant (*in* 751), which lacks functional protein products from both the E1a and

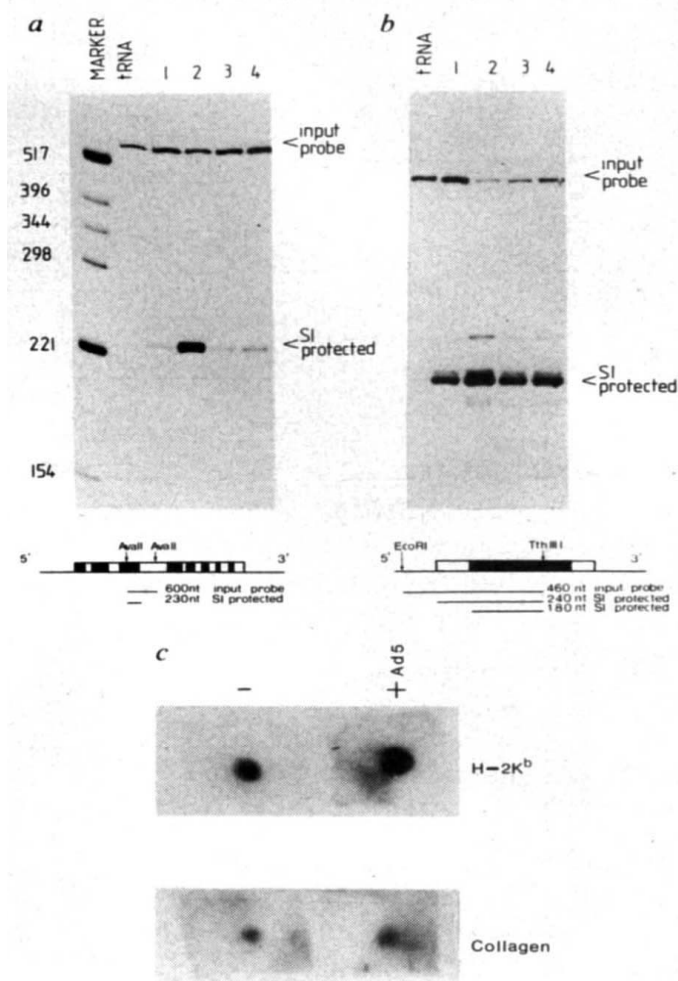


Fig. 1 a, b, *S₁* nuclease analysis of *H-2K^b* (a) and histone H4 (b) mRNA from C57BL/10 mock-infected (lane 1), Ad5-infected (lane 2), *dl312*-infected (lane 3) and *dl313*-infected (lane 4) mouse embryo cells. Markers are ³²P-labelled pBr 322XHinf. c, Dot hybridization of labelled nuclear transcripts from mock-infected (-) and Ad5-infected (+) C57BL/10 mouse embryo cells to *H-2K^b* and type IV collagen gene fragment.

Methods. a, A 600-nucleotide probe spanning the exon III/intron III boundary of the *H-2K^b* gene was end-labelled with reverse transcriptase and hybridized with 15 µg total RNA at 60 °C for 12 h in 10 µl of 80% formamide, 40 mM PIPES pH 6.4, 1 mM EDTA, 400 mM NaCl. The mixture was digested with 3,000 units of *S₁* nuclease (Boehringer) in 300 µl of 300 mM Na-acetate pH 4.8, 200 mM NaCl, 2 mM ZnSO₄ at 20 °C for 1 h, followed by 40 °C for a further hour. *S₁* nuclease-protected DNA was ethanol-precipitated, denatured and electrophoresed on a 7 M urea/7% acrylamide gel^{30,31}. b, A 460-nucleotide probe spanning the 5' end of the histone H4 gene was end-labelled with T4 polynucleotide kinase and hybridized with 15 µg total RNA at 52 °C as described for a. Samples were digested with *S₁* nuclease for 2 h at 20 °C, then treated as for a. c, Nuclei were isolated from mock-infected and Ad5-infected cells. RNA synthesized in 5×10^6 isolated nuclei was labelled for 20 min with 200 µCi [α -³²P]UTP (3,000 Ci mmol⁻¹) in a 300 µl reaction volume as described in ref. 32. Plasmids containing the *H-2K^b* gene and the type IV collagen gene were linearized by digestion with *Hinf*I and *Eco*RI respectively. DNA (15 µg) was bound to a 0.45-µm nitrocellulose filter³³ and hybridized to labelled RNA from 2×10^6 isolated nuclei in a 400-µl volume³². After 46 h, hybridized filters were washed at 54 °C in 0.1 × S.S.C., 0.1% S.D.S. for 45 min, air-dried and exposed for 2 h.

and 13S mRNAs because of a stop codon in the first exon, did not activate the *H-2K^b* gene (Fig. 2a, lane 8). Similarly, infection with a non-tumorigenic Ad12 mutant (*hr1121*) which does not produce the 52K (relative molecular mass 52,000) E1b protein¹⁰ did not induce *H-2K* gene expression (Fig. 2a, lane 9). Infection with a weakly tumorigenic Ad12 E1a mutant

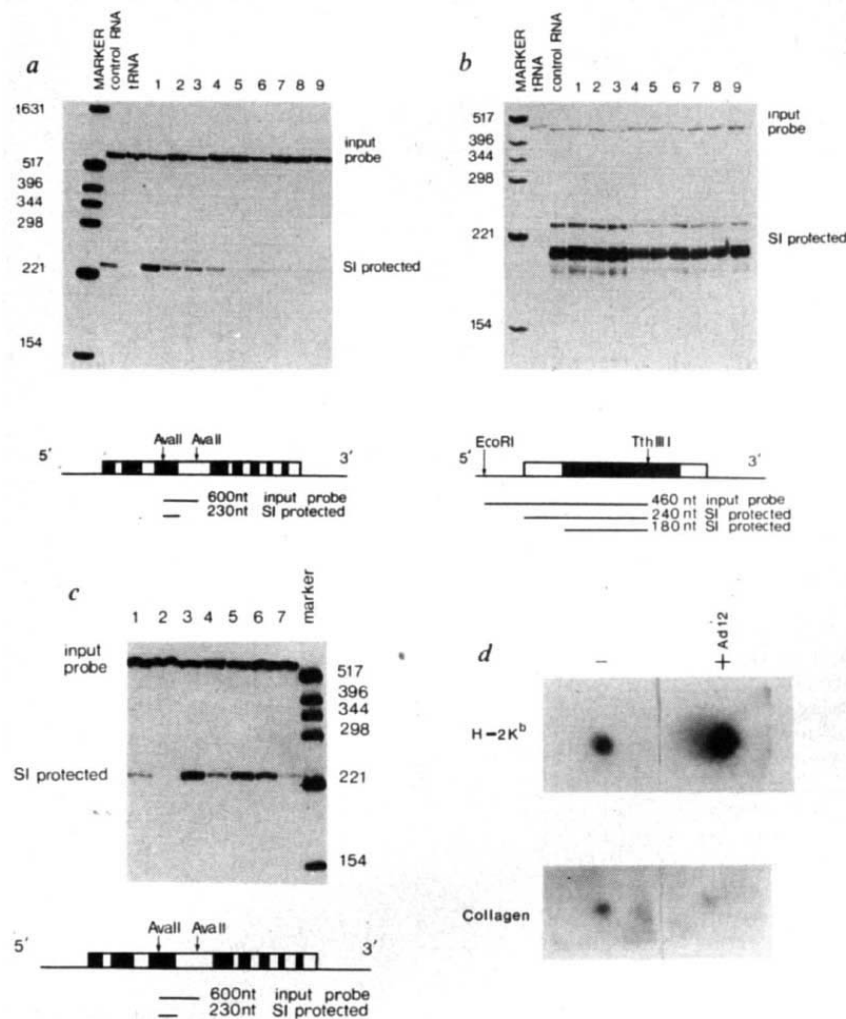
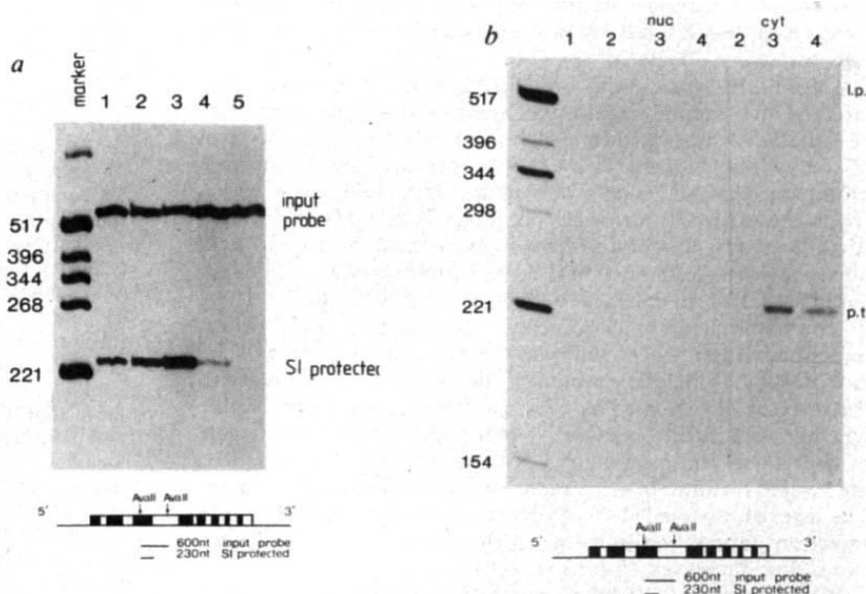


Fig. 2 *a, b*, S_1 nuclease analysis of $H-2K^b$ (*a*) and histone H4 (*b*) mRNA from mock-infected (lane 9) C57BL/10 cells and from cells infected with 10–20 PFU per cell of Ad12 E1a mutant *hr751* (lane 8), Ad12 E1a mutant *hr771* (lane 7), Ad12 E1a mutant *in600* (lane 6), Ad12 E1b mutant *hr1121* (lane 5), *in751* plus *hr1121* (lane 3), *hr751* plus *in600* (lane 4), *in600* plus *hr1121* (lane 2) and Ad12 wild type (lane 1). S_1 nuclease analysis was done as described for Fig. 1*a, b, c*. S_1 nuclease analysis of $H-2K^b$ mRNA from mock-infected C57BL/10 cells (lane 1), control tRNA (lane 2) and from cells infected with Ad12 (lane 3), *in751* (lane 4), *hr771* (lane 5), *in600* (lane 6) and Ad12 *hr1121* (lane 7) at a titre of 50 PFU per cell. S_1 nuclease analysis was done as described for Fig. 1*a, d*. Dot hybridization of labelled nuclear transcripts from mock-infected (–) and Ad12-infected (+) C57BL/10 mouse embryo cells to $H-2K^b$ and type IV collagen gene fragments. Methods as for Fig. 1*c*.

Fig. 3 *a*, S_1 nuclease analysis of $H-2K^b$ gene transcripts from mock-infected C57BL/10 mouse embryo cells (lane 4), control tRNA (lane 5) and from cells infected with polyoma virus (lane 1) or SV40 (lane 2) at a multiplicity of infection of 10–50 PFU per cell. Lane 3 contains control RNA isolated from C57BL/10 cells treated with $1,000 \text{ U ml}^{-1}$ mouse γ -interferon. *b*, S_1 nuclease analysis of cytoplasmic (cyt) and nuclear (nuc) $H-2K^b$ mRNA from mock-infected (lane 2), Ad5-infected (lane 3, 10 PFU per cell) and Ad12-infected (lane 4, 10 PFU per cell) C57BL/10 mouse embryo cells. Lane 1 contains a tRNA control. S_1 nuclease analysis was carried out as described for Fig. 1. All lanes contain an equal amount of RNA (20 μg).



(*hr771*) which produces some E1a protein products only after a delay in permissive cells¹⁰, or with an Ad12 E1a mutant (*in600*) which contains the normal E1a 12S RNA and a truncated 13S RNA¹⁰ resulted in, respectively, no increase (Fig. 2*a*, lane 7) or only a marginal increase (lane 6) in the level of $H-2K$ gene transcripts at the multiplicity of infection used (10 plaque-forming units (PFU) per cell). Co-infection of E1a mutants *hr751* and *in600* with each other or with the *hr1121* E1b mutant resulted in an increase in the level of $H-2K$ mRNA (Fig. 2*a*, lanes 4, 3 and 2 respectively); this effect could be caused by

either complementation or an increased number of virus particles (10 PFU per cell of each; see below). The levels of histone H4 transcripts in these infections were only 1.5–2 times higher than in mock-infected cells (Fig. 2*b*).

Failure of adenovirus E1a mutants to activate the $H-2K^b$ gene may result from the lack of production of other adenovirus early proteins which are normally activated by the E1a gene products^{11,12}. We therefore repeated the Ad12 mutant infections at a higher multiplicity of infection (50 PFU per cell) to allow production of other adenovirus early proteins in the absence of

the E1a proteins (ref. 13 and R. Grand and P. Gallimore, personal communication). As shown in Fig. 2c, infection with a high titre of E1a mutants (*in600* and *hr771*) resulted in activation of the *H-2K^b* gene equal to that effected by wild-type virus. This result indicates that it is not the protein products of the E1a 13S transcript alone that activate the *H-2K^b* gene. The E1b 52K protein and the products of the E1a 12S RNA, or 13S exon 1 RNA, are directly or indirectly necessary for *H-2K^b* gene activation since high-titre infection with either the E1b 52K mutant (*hr1121*) or the E1a mutant which lacked protein products of both 12S and 13S RNAs (*hr751*) failed to significantly (less than twofold) increase the level of *H-2K^b* gene transcripts (Fig. 2c). Measurement of the relative rate of transcription of the *H-2K^b* gene in mock-infected and Ad12-infected cells indicated that the increase in the level of *H-2K* mRNA following viral infection was at least in part due to transcriptional activation (Fig. 2d).

This result may reflect the *trans*-acting enhancing properties of the E1a protein products, which can activate viral^{11,12,14} newly introduced¹⁵⁻¹⁸, integrated¹⁹ and endogenous genes²⁰ either directly or through inactivation of cellular repressor proteins^{13,21}. Alternatively, the gene activation may be mediated by a general anti-viral state induced by cellular proteins in response to either viral replication or viral proteins^{21,22}; this seems unlikely, as Ad12 virus infections at 10 PFU per cell did not result in detectable levels of interferon (<4 U ml⁻¹) in the cell culture (data not shown).

The results presented here indicate that infection of mouse embryo cells with either Ad5 or Ad12 results in transcriptional activation of the MHC *H-2K* gene. Viral protein products from the 12S E1a mRNA or the first exon of the E1a gene, which is common to both 13S and 12S mRNAs, and the 52K E1b protein are necessary for this activation (Fig. 2). As yet, we do not know whether viral protein products from both the E1a and E1b regions are mediating the activation directly or via interaction with other (early) viral or cellular proteins. For example, the Ad5 E1b 58K protein binds the same p53 cellular protein as simian virus 40 (SV40) large-T antigen²⁴. To determine whether other DNA tumour viruses such as SV40 mediate induction of the class I MHC genes using similar mechanisms²⁵ and whether induction is related to permissiveness of the virus/cell system, we infected mouse embryo cells with either SV40 (non-permissive, as is adenovirus) or polyoma virus (permissive). Infection with either of these viruses resulted in activation of the *H-2K* gene but at a lower level than that resulting from the adenovirus infections, which itself was lower than that induced by interferon in a control experiment (Fig. 3a).

Our results are in contrast to those of Schrier *et al.*⁵ who reported a decrease in the level of cytoplasmic class I mRNA and suppression of MHC antigen expression in Ad12-, but not in Ad5-infected cells. As Schrier *et al.*⁵ measured cytoplasmic mRNA, the difference could be due to a block in the transport of the newly synthesized mRNA from the nucleus to the cytoplasm. To test this possibility, the cells were infected with Ad5 and Ad12 (10 PFU per cell) and both the nuclear and cytoplasmic mRNA were quantitated. The results (Fig. 3b) show that the *H-2K* mRNA in the cytoplasm is increased between 10 and 15-fold in both cases, while the mRNA level in the nucleus is much lower and only visible after a longer exposure, which excludes the possibility of a block in mRNA transport.

However, preliminary experiments using radioimmune labelling of the Ad5- and Ad12-infected cells indicate that the amount of *H-2K^b* antigen on the cell surface increases only marginally, if at all, despite the large increase in cytoplasmic *H-2K^b* mRNA (data not shown). Thus, the results suggest that infection first induces a stimulation of MHC transcription, which normally would enable a better cellular immune response against infected cells. In the case of viruses, for example Ad5 and Ad12, this may not result in an increased expression of MHC antigens on the cell surface because of other viral factors²³. In the case of oncogenic viruses such as Ad12, this may be followed by a suppression of MHC class I gene transcripts to allow escape

from immune surveillance. Whether this is caused by an immunity from natural killer cells²⁶⁻²⁸ alone and/or from cytotoxic T cells⁶ as part of an MHC-restricted response²⁹, is unclear.

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Irreversible swelling of secretory granules during exocytosis caused by calcium

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The fusion of the limiting membrane of a secretory granule with the plasmalemma during exocytosis is equivalent to the fusion and release of contents that occurs when phospholipid vesicles fuse with planar bilayers^{1,2}. Experiments with bilayers demonstrate that phospholipid vesicles must swell if they are to fuse³. Also, inhibition of exocytosis in solutions of high osmolality occurs in several types of secretory cell⁴⁻⁸. We report here experiments on the cortical granule exocytosis of sea-urchin eggs. Exocytosis is prevented when the osmolality of the medium surrounding the eggs is raised from 1 to 2 osmol kg⁻¹. High osmolality also prevents calcium-dependent exocytosis *in vitro*. Prior treatment with calcium at high osmolality triggers fusion when normal osmolality

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is restored, even if calcium is removed before dilution. Addition of calcium causes the cortical granules to swell. The large increase in membrane capacitance which normally accompanies fusion⁹ is absent in eggs activated in solutions of high osmolality. Our data are consistent with the idea that a secretory granule must swell to fuse with the plasma membrane and support the hypothesis^{2,10} of an osmotically driven fusion step during exocytosis.

When a sea-urchin egg is fertilized or activated with the calcium ionophore A23187, there is a rapid and complete exocytosis of the cortical granules that lie immediately beneath the plasmalemma^{11,12}. This secretory event is stimulated by an increase in cytoplasmic free calcium¹³. If eggs are treated with calcium ionophore in sea water to which sucrose, sodium sulphate or stachyose is added to produce an osmotic pressure increase corresponding to 1 osmol per kg, they retain their full complement of cortical granules (Fig. 1). The inhibition is reversible in that eggs returned to sea water after treatment with ionophore in the presence of high-osmolality solution undergo

a complete exocytosis. Pretreating the eggs with osmoticant and returning them to sea water before adding calcium ionophore does not prevent exocytosis. The similar effects of 0.7 M stachyose, 0.55 M sodium sulphate and 0.85 M sucrose suggest that it is the osmotic rather than the pharmacological properties of the three substances which are responsible for the inhibition of secretion. In the presence of solutions of high osmotic pressure the granules shrink, suggesting that if swelling of the granule is hindered then exocytosis is inhibited.

We wished to determine whether solutions of high osmolality were indeed inhibiting the fusion of the secretory granule membrane with the plasma membrane or whether fusion had, in fact, taken place. Under normal conditions the granule contents take up water and salts as they disperse^{14,15}. It was possible that the granule and plasma membranes had fused with the formation of an exocytotic stoma and that solutions of high osmolality were preventing granule dispersal.

One consequence of exocytosis of the cortical granules of the egg is a two- to threefold increase in the membrane capacitance which is the result of addition of cortical granule membrane to the plasmalemma^{9,16}. Figure 2 shows that under conditions in which exocytosis (by which we mean both membrane fusion and the dispersal of the granule contents) is prevented, only a very small increase in membrane capacitance is observed, ~10%

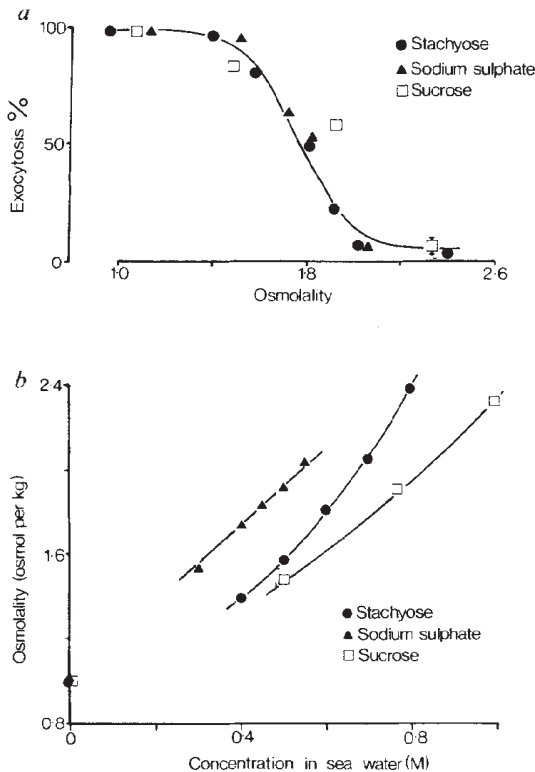


Fig. 1 *a*, Effect of increasing osmolality on cortical granule exocytosis in *Lytechinus pictus* eggs. Jelly-free eggs were resuspended in artificial sea water¹⁹ (1.02 osmol per kg) or filtered sea water (0.90 osmol per kg) containing various concentrations of sucrose, stachyose or sodium sulphate. *b*, Osmolality was measured using a Wescor vapour pressure osmometer. Resuspended eggs were mixed with an equal volume of solution of identical composition containing 20 μ M A23187 (Calbiochem), and 5 min after mixing, the extent of exocytosis of the cortical vesicles was assessed in the light microscope by gently flattening the eggs against a coverslip. The cortical granules are identified easily by their size and degree of association with the egg cortex. The granules in a disk of 50 μ m radius were counted in 10 eggs selected at random. Each experiment was recorded on photographic film (Zeiss, Nikon or Leitz DIC optics with $\times 100$, 1.25 or 1.4 NA objectives). The osmolality of the solutions being tested was not known to the observer. Inhibition by stachyose solutions was time-dependent. After resuspension in stachyose the eggs shrank rapidly then swelled slowly towards their original size. Exocytosis eventually occurred at all stachyose concentrations tested. Stachyose did not merely reduce the rate of exocytosis, as in 0.8 M stachyose sea water, for example, no detectable exocytosis occurred for 25 min after addition of the calcium ionophore, although exocytosis was extensive at 30 min after addition. In untreated preparations, exocytosis is complete in 1 min. Temperature, 20 $^{\circ}$ C.

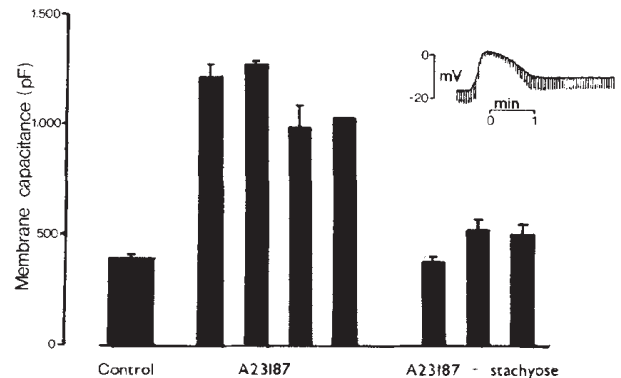
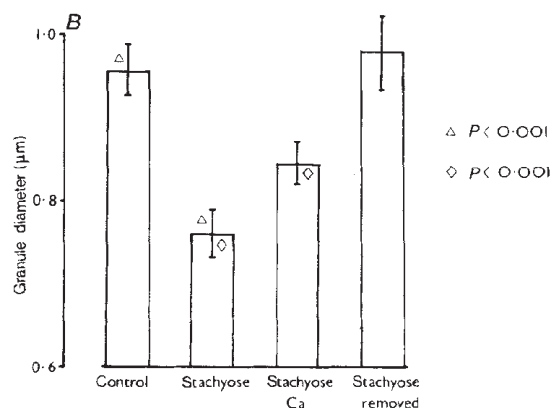
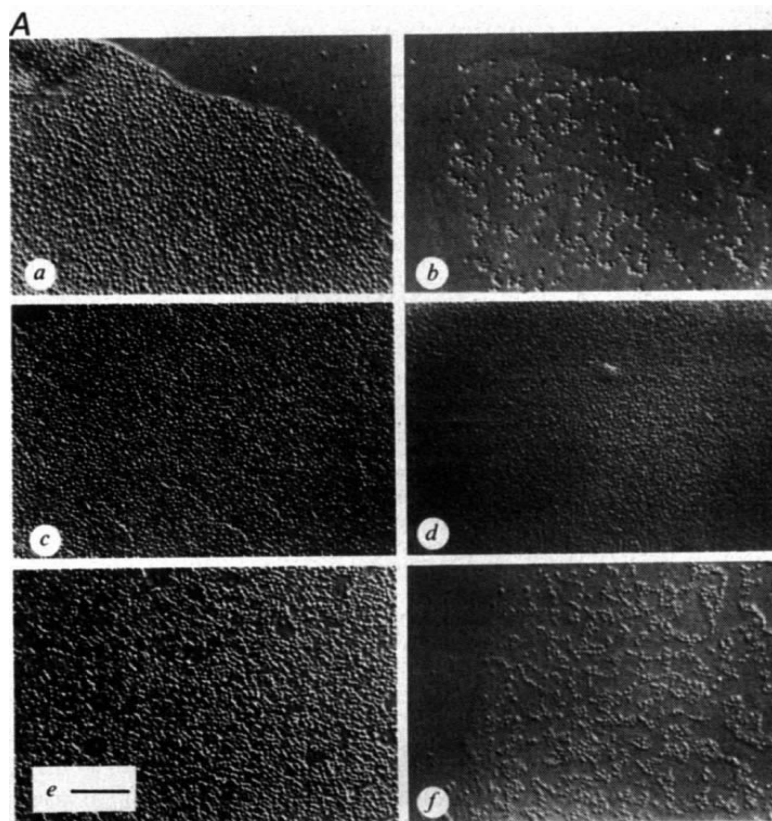


Fig. 2 Effect of stachyose on the increase in egg membrane capacitance resulting from membrane fusion. The inset shows the membrane potential alterations when the calcium ionophore A23187 (50 μ M in ethanol) is added in the presence of 0.8 M stachyose in artificial sea water (2.39 osmol per kg). Membrane capacitance was measured by injecting constant current pulses through one microelectrode while recording membrane potential with a second microelectrode. Data were recorded on magnetic tape and the membrane time constant and resistance measured from five consecutive current pulse records made from tape with a chart recorder, shown (A23187 and A23187 + stachyose) as single experiments (mean $\pm$ s.e.m.). Controls are shown as mean and s.e.m. of 22 estimates of membrane capacitance in six unfertilized eggs. The rise time of the recording system was 3.5 ms. Egg input resistance before activation, 66–180 M Ω ; after activation, 20–60 M Ω ; current pulse, 100 or 200 pA; electrode resistance, 50–100 M Ω (KCl-filled). Temperature, 16 $^{\circ}$ C. The small increase in membrane capacitance observed when eggs are treated with A23187 in the presence of 1 M stachyose (10% of the change seen in control eggs) may result from exocytosis of 10% of the granules. The converse assumption is that it results from a decrease in the membrane resistance (R_s) between the interior of each granule and the external solution. The decrease in R_s could be distributed across the area of contact of granule and plasma membranes or could result from the appearance of a small aqueous pore or channel between the interior of each granule and the external medium. Our data indicate that the capacitance of the granule membrane (C_g) is 800 pF and that of the cell membrane (C_m) 400 pF. We have assumed that the granule membrane resistance (R_g) and granule membrane capacitance are linked by series resistance R_s to the plasma membrane resistance (R_m) and capacitance. Thus, $I = ((R_s + R_g + R_m)/R_m R_g + R_s) V + ((C_m R_m R_g + R_s) + C_g R_g (R_m + R_s))/R_m (R_g + R_s) dV/dt + (C_g C_m R_s R_m R_g / R_m (R_g + R_s)) d^2V/dt^2$. I , membrane current; V , membrane potential; t , time. Solving for V and t with a step change of membrane current we find from the apparent increase in membrane time constant in the presence of 1 M stachyose that if R_m is ≤ 200 M Ω then R_s cannot be < 50 M Ω . This sets an upper limit on the size of the pores. There are $\sim 30,000$ cortical granules in a *L. pictus* egg⁸, which gives a resistance per pore of $1.5 \times 10^{12} \Omega$. This resistance is of the order of the resistance of the smallest ion channels so far discovered in biological membranes; we therefore believe that fusion and the formation of an exocytotic stoma have occurred in $\leq 10\%$ of the cortical granules of the egg under these conditions.

Fig. 3 A, Effects of stachyose on cortical vesicle exocytosis *in vitro*. Cortical fragments consisting of vitelline coat plasma membrane and attached cortical vesicles were prepared by attaching eggs by their vitelline coat to glass coverslips with polylysine and shearing away the bulk of the egg using a jet of isolation medium (220 mM potassium glutamate, 500 mM glycine, 5 mM $MgCl_2$, 10 mM NaCl, 2.5 mM ATP, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulphonyl fluoride, 1 mM EGTA, pH 6.7; ref. 19). Cortical fragments on the right (**b**, **d**, **f**) have been treated with calcium ($6 \mu M$; $Ca/EGTA = 0.838$; ref. 19). **a**, Cortical fragment in isolation medium; **b**, fragment in isolation medium 2 min after addition of $6 \mu M$ calcium; **c**, fragment in isolation medium containing 1 M stachyose showing granules of decrease size; **d**, fragment in isolation medium containing 1 M stachyose 5 min after treatment with $6 \mu M$ calcium showing inhibition of exocytosis of cortical vesicles; **e**, fragment returned to isolation medium after treatment with 1 M stachyose-containing isolation medium (vesicles swell to their original size and will undergo exocytosis if exposed to calcium, not shown); **f**, fragment returned to isolation medium after treatment for 2 min with 1 M stachyose-containing medium containing $6 \mu M$ Ca, then for 2 min with 1 M stachyose-containing medium ($<10^{-8}$ M Ca) before returning to isolation medium ($<10^{-8}$ M Ca); exocytosis occurs in the absence of calcium when stachyose is removed. As in whole eggs, the inhibition of exocytosis is time dependent. Exocytosis occurred in the presence of stachyose after 15–20 min. At normal osmolarities, exocytosis occurs completely within 1 min of calcium addition. Temperature, $20^\circ C$; scale bar, $10 \mu m$. **B**, Effects of stachyose and calcium on the size of cortical vesicles *in vitro*. Control, preparation in isolation medium; Stachyose, in 1 M stachyose-containing medium; Stachyose Ca, in 1 M stachyose-containing isolation medium containing $6 \mu M$ calcium; Stachyose removed, preparation treated with 1 M stachyose-containing medium and returned to stachyose-free medium. Photomicrographs similar to those shown in **A** were printed at $\times 10,000$ and the diameter of 100 granules estimated from the measurements of granule perimeter made using a digitizing tablet. Mean diameter $\pm s.e.m.$ are shown. The statistical significance of the differences in mean diameter were assessed using a one-tailed Kolmogoroff-Smirnoff test. The dispersion in measured granule diameters seems to result from the dispersion of apparent granule sizes and not from errors in measurement, as the measuring procedure was precise, giving granule diameters of 0.98 ± 0.038 and $0.98 \pm 0.041 \mu m$ (mean $\pm s.e.m.$) in two separate experiments in which granules were treated with high-osmolarity stachyose and returned to normal osmolarity.



of that seen at normal osmolarity. Under these conditions, it seems that no exocytotic stoma form and that inhibition of granule swelling prevents fusion as well as dispersal. These experiments also indicate that stachyose is not preventing the ionophore-induced internal calcium transient, as we observe the calcium-associated membrane depolarization.

Inhibition of exocytosis by applying solutions of high osmolarity to the outside of the egg may be the consequence of alterations in ionic strength or viscosity in the cytoplasm caused by the shrinkage of the egg. Therefore, we investigated the effects of high osmolarity on exocytosis *in vitro*.

A preparation consisting almost entirely of plasma membrane with attached cortical granules can be made by attaching eggs to glass coverslips with polylysine and shearing away the bulk of the egg¹⁷. This preparation gives access to the cytoplasmic face of the plasma membrane. Granules undergo exocytosis in response to micromolar calcium concentrations^{18–20}. It is known that high-osmolarity sucrose solutions reduce the rate of exocytosis *in vitro* and that under these conditions the effect of calcium seems to be irreversible²¹. Inhibition by sucrose is

incomplete because the cortical granules have a substantial sucrose permeability, so we used stachyose, a tetrasaccharide to which the granules are less permeable. Figure 3A shows the result of an experiment in which calcium was added to the preparation in the presence of stachyose (1 M in isolation buffer; 3 osmol per kg) and in which exocytosis is prevented. If calcium is removed and the preparation is allowed to remain for 5 min in EGTA-containing 1 M stachyose isolation buffer before dilution into isolation buffer containing EGTA, then exocytosis occurs on dilution to normal osmolarity. Exposing a preparation to stachyose in the absence of calcium and returning it to normal isolation medium does not result in a substantial loss of cortical granules; >90% are unaffected by this treatment. These experiments demonstrate that, even in the presence of 1 M stachyose, calcium is exerting an effect whose results persist after its removal. That is, the calcium-dependent step in exocytosis is irreversible in stachyose-treated cortices.

The planar array of cortical granules on the isolated plasma membrane forms an excellent optical image. Figure 3B shows that granules shrink considerably when exposed to 1 M

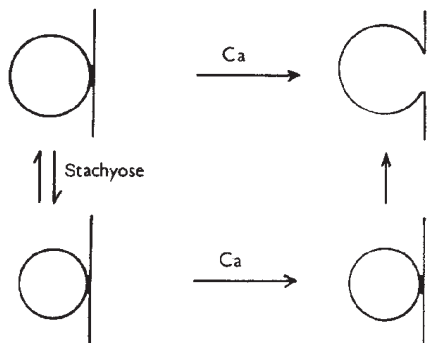


Fig. 4 Diagrammatic representation of the changes thought to be occurring in our experiments. Calcium promotes swelling and exocytosis whereas stachyose and other osmoticants cause reversible shrinkage of the cortical vesicles. If calcium is added in the presence of stachyose, the vesicle swells slightly but the swelling is insufficient to cause fusion. However, the state of the vesicle is altered irreversibly by calcium. Removal of stachyose after treatment with calcium leads to exocytosis.

stachyose in isolation medium and appear to swell slightly when they are also exposed to calcium. We suppose that calcium-induced swelling can only cause exocytosis if granules swell beyond their usual size.

Cortical granule exocytosis *in vitro* is not affected by large increases in ionic strength. We find that it occurs in solutions containing 1 M KCl, which is four times the ionic strength of cytoplasm²². Nor is a solution of high viscosity (30% Ficoll, relative molecular mass 400,000, 96 cP) inhibitory *in vitro*²¹. Therefore, it seems likely that high osmolality prevents exocytosis in whole eggs, as it does *in vitro*, by preventing granule swelling^{21,23}. We find that higher concentrations of stachyose are needed to inhibit exocytosis *in vitro* (1 M in isolation buffer; 3.0 osmol per kg) than in whole eggs (0.7 M in sea water; 2.2 osmol per kg). We attribute this to a small but significant permeability of the granule membrane to stachyose, because a proportion (~10%) of the granules burst when returned to normal osmolality after treatment with stachyose in the absence of calcium. This interpretation is supported by the observation that inhibition of exocytosis by stachyose is time-dependent (see Figs 1, 3 legends).

A diagram consistent with our results is shown in Fig. 4, in which calcium induces a swelling of the secretory granule which under normal circumstances leads to fusion. In the presence of solutions of high osmolality, calcium-induced swelling is insufficient to cause fusion. But calcium has irreversible effects and fusion occurs when the granule swells further on exposure to normal osmotic pressures. We do not know why the cortical granule swells on exposure to calcium, nor why granule swelling leads to fusion. Granule swelling may result from an increased influx of ions and water across the granule membrane as a consequence of calcium-induced permeability changes or as a consequence of calcium-induced alterations of the osmotic properties of the granule contents. Fusion may occur because the granule ruptures preferentially at its point of contact with the plasma membrane, as has been suggested to explain the fusion of phospholipid vesicles with planar bilayers²⁴. A mechanism in which calcium stimulates an ion exchange across the apposed granule and plasma membranes²⁵ seems to us less likely because exocytosis in sea-urchin eggs will occur in the absence of external monovalent ions²⁶.

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Movement of myosin-coated beads on oriented filaments reconstituted from purified actin

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Although the biochemical properties of the actin/myosin interaction have been studied extensively using actin activation of myosin ATPase as an assay^{1,2}, until recently no well-defined assay has been available to measure the mechanical properties of ATP-dependent movement of myosin along actin filaments. The first direct measurements of the rate of myosin movement *in vitro*^{3,4} used a naturally occurring, biochemically ill-defined array of actin filaments from the alga *Nitella*. We report here the construction of an oriented array of filaments reconstituted from purified muscle actin and the use of this array in a biochemically defined quantitative assay for the directed movement of myosin-coated polystyrene beads. We demonstrate for the first time that actin alone, linked to a substratum by a protein anchor, is sufficient to support movement of myosin at rates consistent with the speeds of muscle contraction and other forms of cell motility.

We have used severin, a protein from the amoeba *Dictyostelium*^{5,6} which binds with very high affinity to the barbed end of actin filaments⁷ (Fig. 1a). Using biotinylated severin, actin filaments can be bound to avidin immobilized on a carbon-coated electron-microscope grid, which serves as an optically clear substratum (Fig. 1b). In appropriate flow conditions, the bound filaments should orient with their free pointed ends in the downstream direction (Fig. 1c). According to H. E. Huxley's model⁸, myosin moves along actin from the pointed end to the barbed end. Therefore, myosin beads placed on the actin/severin/avidin array should move upstream against the flow of buffer. The use of electron-microscope grids allows the actin array to be visualized by transmission electron microscopy after measurement of bead movement.

We followed the active movement of 40 myosin beads over distances of 10-50 μm on actin arrays demonstrated by electron microscopy to be markedly oriented (Fig. 2). The average velocity of skeletal myosin beads was $4 \mu\text{m s}^{-1}$ (26 beads, range $0.5-6 \mu\text{m s}^{-1}$), which is close to the measured maximum rate of contraction of intact muscle under zero load ($6 \mu\text{m s}^{-1}$)⁹. The average velocity of *Dictyostelium* myosin beads was $0.6 \mu\text{m s}^{-1}$ (14 beads, range $0.3-1.3 \mu\text{m s}^{-1}$). These velocities measured with

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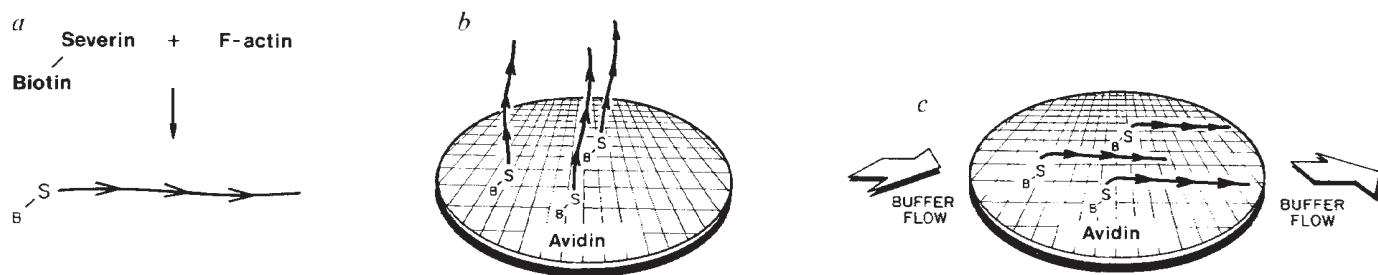
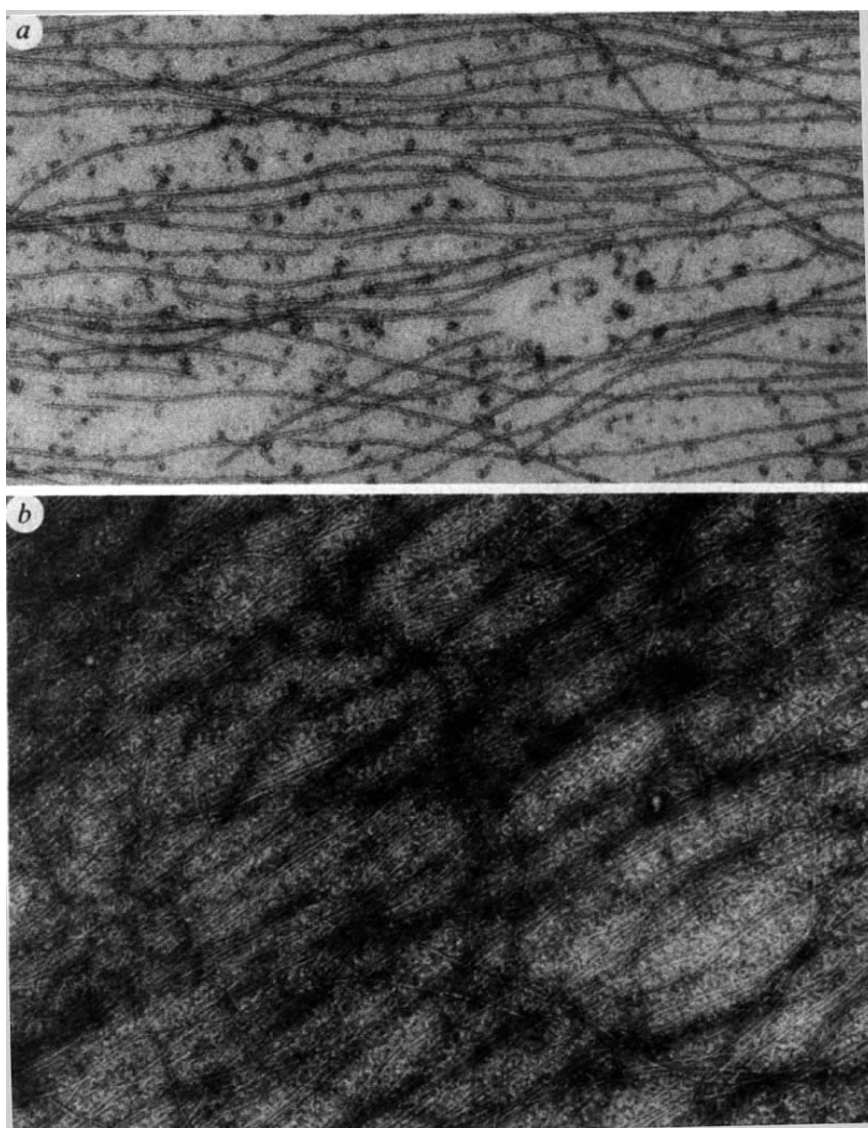


Fig. 1 Schematic representation of the method for preparing the oriented actin substratum. *a*, Severin was biotinylated as follows: 800 μ l severin at 0.25 mg ml⁻¹ in 50 mM sodium phosphate, pH 7.5, was combined with 15 μ l 1 mg ml⁻¹ *N*-hydroxysuccinimido biotin in dimethyl sulphoxide for 90 min at 22 °C, and the reaction quenched with 5 μ l 0.1 M glycyl glycine, pH 7.5. The mixture was dialysed against F buffer (10 mM imidazole, pH 7.0, 25 mM KCl, 4 mM MgCl₂, 0.5 mM 2-mercaptoethanol) containing 4 mM EGTA at 4 °C for 36 h. Severin/biotin was mixed with fresh 0.25 mg ml⁻¹ F-actin¹³ in F buffer with 1 mM ATP and 0.2 mM CaCl₂ to a ratio of severin:actin 1:500 to produce a population of filaments with their barbed ends blocked by severin. *b*, *c*, A carbon-coated electron-microscope grid (200 mesh), coated with 100 μ g ml⁻¹ avidin in 50 mM NaHCO₃, pH 9.5, for 10 min at 22 °C, and washed with F-buffer containing 0.2 mM CaCl₂, was pinned onto the bottom of a channel (3 mm wide, 1 mm deep) moulded in silicone polymer. Severin/actin filaments were applied to the grid surface with laminar flow in the channel at 10 ml min⁻¹ for 10 min. Unattached actin was washed from the grid surface in a flow (5 ml min⁻¹) of F buffer containing 25 μ g ml⁻¹ actin, 4 mM EGTA and 1 mM ATP, leaving a mat of ordered filaments.

Fig. 2 Electron micrographs of F-actin oriented as described in Fig. 1 legend. *a*, Image less densely populated with actin filaments than *b*, revealing the orientation of the filament resulting from the flow. $\times 58,500$. *b*, Image of dense meshwork of oriented filaments taken from a grid that supported movement of myosin beads. $\times 52,000$. To obtain the images shown, grid was carefully removed from the channel noting its orientation with regard to the buffer flow. Aqueous uranyl acetate (1%) was flowed in the same direction across the grid, the excess being drawn off with filter paper from the downstream edge.

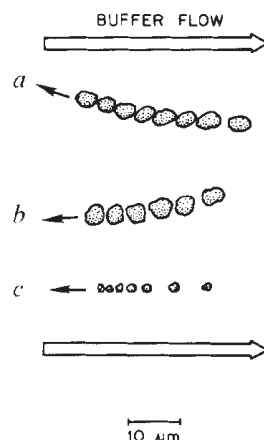


the synthetic actin array are remarkably similar to those observed using the *Nitella*-based assay⁴ (2–6 μ m s⁻¹ for skeletal muscle myosin and 0.5–1.5 μ m s⁻¹ for *Dictyostelium* myosin).

These results show that, to support myosin movement, actin filaments need not be in the form of bundles (as they are in

Nitella), nor are accessory proteins required (other than a filament anchor such as severin). In addition, our results suggest that the specific myosin type (skeletal muscle or *Dictyostelium*) is the principal determinant of bead velocity, rather than the type of actin (muscle or *Nitella*).

Fig. 3 Movement of myosin beads in the upstream direction with respect to the buffer flow. *a, b*, The two larger distinct bead aggregates were coated with $200 \mu\text{g ml}^{-1}$ Dictyostelium myosin and their positions shown every 10 s, corresponding to a rate of movement of $\sim 0.5 \mu\text{m s}^{-1}$. *c*, The smaller bead aggregate was coated with $200 \mu\text{g ml}^{-1}$ skeletal muscle myosin and its position shown every 2 s, corresponding to a rate of movement of $\sim 2 \mu\text{m s}^{-1}$. Myosin beads were deposited on the actin array by microinjection, suspended in F buffer containing 1 mM ATP, 0.2 M sucrose, 1 mM dithiothreitol and 4 mM EGTA. With F buffer containing $25 \mu\text{g ml}^{-1}$ actin, 4 mM EGTA and 1 mM ATP flowing in the channel at 0.04 ml min^{-1} , myosin beads and distinct bead aggregates attached to the grid surface and moved upstream. Myosin bead preparation and analysis of movement were as described previously⁴. The diagram shows typical bead movements. To be scored as an active event, bead movements had to be: (1) upstream to the direction of buffer flow; (2) at the plane of focus of the surface of the grid; (3) associated with a distinct binding event; (4) characterized by sliding rather than tumbling. In several cases, the rate of buffer flow was changed in the course of active bead movement and no effect on the rate of bead movement was observed. In contrast, movements resulting from bulk flow were: (1) downstream; (2) generally not in the plane of focus of the surface of the grid; (3) not associated with binding to the grid surface; (4) characterized by tumbling when the beads were close to the surface of the grid; (5) dependent on the presence and rate of buffer flow.



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Compartmentalization of sickle-cell calcium in endocytic inside-out vesicles

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Much recent interest in the mechanism of dehydration of the dense subpopulation of sickle-cell anaemia (SS) red cells, including the 'irreversibly sickled cells' (ISCs), stems from the view that these relatively rigid cells have a major role in the two main clinical features of the disease, namely haemolytic anaemia and microvascular occlusion¹. The discovery that SS red cells have an elevated calcium content and accumulate Ca^{2+} during deoxygenation-induced sickling²⁻⁴ suggested a working hypothesis of wide appeal for the mechanism of cell dehydration: retained calcium would activate the red cell Ca^{2+} -sensitive K^+ channels⁵, causing progressive net loss of KCl and water⁶⁻⁸. However, retained calcium, which seemed as weakly bound to cytoplasmic buffers as in normal red cells⁹, failed to show any measurable activation of K^+ channels or Ca^{2+} pumps in metabolically normal SS cells, despite the apparent functional normality or near-normality of these transport systems⁹⁻¹⁷. We now offer a possible explanation for this failure. We show that, contrary to the traditional views, SS cells, and to a lesser extent normal human red cells, possess intracellular vesicles with ATP-dependent Ca^{2+} -accumulating capacity, and that nearly all the measurable calcium of fresh SS cells is contained within such vesicles, probably in the form of precipitates with inorganic or organic phosphates.

Intracellular vesicle-like structures have been described previously in electron micrographs of single sections of fixed normal red cells^{18,19}. In such sections, however, true vesicles are indistinguishable from artefacts resulting from occasional membrane infoldings. To demonstrate that the vesicles are fully contained within the cell, it is necessary to examine the three-dimensional structure of a representative number of cells in serial section. Figure 1 shows typical runs of consecutive sections of fresh SS cells and ghosts chosen to illustrate genuine and artefactual vesicular images. Analyses of 75 SS cells, 59 SS ghosts and similar studies (not shown) with 36 normal red cells and 62 normal ghosts, revealed that both SS and normal red cells possess fully enclosed vesicles. In at least 84% of the normal cells, one to four tiny vesicles were found, which were $\sim 0.1 \mu\text{m}$ in diameter. The vesicles were usually contained within one or two consecutive $0.10\text{--}0.15\text{-}\mu\text{m}$ sections, but occasionally extended over up to seven such consecutive sections, with tubular or

Drag on a sphere in laminar flow at low Reynolds's number is directly proportional to the bulk fluid velocity. As discussed previously³, sufficient power is generated by a single myosin molecule to move a $1\text{-}\mu\text{m}$ bead $5 \mu\text{m s}^{-1}$ upstream against a flow of $5 \mu\text{m s}^{-1}$. Because the flow rate near the beads in the present experiments is $\leq 1 \mu\text{m s}^{-1}$ (determined by measuring the velocity of unattached beads moving downstream near the substratum), it is probable that the limit on the speed of myosin movement over actin in this assay is determined by the kinetics of the biochemical reaction. The finding that some beads move slower than this maximum may reflect the presence of disorder in the array of actin filaments. Two observations are consistent with the presence of local regions of disorder. Only one-third of the myosin beads that attach to the substratum move, and those move over distances of $10\text{--}50 \mu\text{m}$ and stop, apparently tightly bound to the electron microscope grid.

Several biochemically defined actin/myosin-based *in vitro* models of cell motility have been suggested¹⁰⁻¹², but none of these has offered a quantitative measure of myosin movement. Many types of experiment are now possible with the biochemically defined assay described in the present study. One important application will be in the identification and purification of factors that regulate actin/myosin-mediated cell motility. This assay can also be used to study the effects of genetic manipulation of both actin and myosin structure on myosin movement along actin filaments. It should be possible to locate and define the amino-acid residues critical for the transduction of chemical energy liberated by ATP hydrolysis into the mechanical work of cellular motility.

We thank the NIH (grants GM30387 and GM33289 to J.A.S.) and the American Heart Association (M.P.S. is an American Heart Association Established Investigator) for financial support. S.J.K. is a trainee of the Medical Scientist Training Program at Stanford Medical School, supported by NIH grant GM07365. *Note added in proof:* We have also observed movement on actin filaments applied by flow to severin nonspecifically bound to an electron-microscope grid surface.

Fig. 1 Genuine and artefactual vesicular images. The cell in *a*, which is not an ISC, illustrates the appearance of a genuine, fully-enclosed vesicle (near the centre) containing some electron-dense material, as well as a long tubular artefact with open communication to the medium (on the right). The cell in *b* is part of an ISC and illustrates the complex structure of a large vesicle also containing electron-dense material. The images in *c* are from an SS ghost. The mottled background is characteristic of SS cells alone and may be caused by haemoglobin (Hb) aggregates. (Following hypotonic lysis, SS ghosts viewed under phase microscopy contained many dense aggregates which moved about within the ghosts; these were rare and much smaller in normal ghosts, and probably represent clumps of denatured Hb.) Many of the vesicles seen in the SS ghosts also contain, or are associated with, electron-dense material. The relative vesicular volume in individual ghosts was estimated by weighing cut-outs of photocopies of the serial sections of SS ghosts and the vesicles contained within them; it varied from <0.02% to 19%, with typical values of ~0.1–0.4%. The high electron density of the cytoplasm and vesicle-associated precipitates, as well as the convoluted shape of most vacuolar spaces, precludes such estimates in the serial sections of intact SS cells.

Methods. Blood was drawn from normal subjects and from sickle-cell anaemia patients, into heparinized containers. Only SS cells are shown in this figure. The red cells were washed four times in saline and packed at 1,200g for 1 min in small microfuge tubes. 20 μ l of packed cells was fixed by addition to 1 ml of buffer containing 2.5% glutaraldehyde and (in mM): KCl, 75; NaCl, 75; HEPES-K (pH 7.2), 10. After 30 min at room temperature, the cells were washed twice with buffer alone. The cell pellets were post-fixed in 1% osmium tetroxide, dehydrated in graded ethanol solutions and embedded in Araldite resin. Serial sections (0.10–0.15 μ m) were cut, mounted on Pioloform support films and slotted grids and stained with lead citrate²⁶. Similar images were obtained with red cells fixed in 0.15 M cocodylate buffers, or by using 1% osmium tetroxide with or instead of glutaraldehyde. With all solutions, similar low proportions of deformed cells, such as those illustrated in *a*, were seen with preparations of SS cells but not with normal cells. Phase-contrast inspection of specimens before and after fixation did not reveal major differences other than in the overall rigidity of fixed cells. Ghosts were prepared by lysing packed cells in 50 vol. of ice-cold medium as described in Fig. 4 legend. Intact red cells were packed and 20 μ l transferred to microfuge tubes containing 1 ml of lysing medium with 1% osmium tetroxide. After 1 h at room temperature, the ghosts were washed once with lysing medium alone, and the pellets processed as for the intact cells.

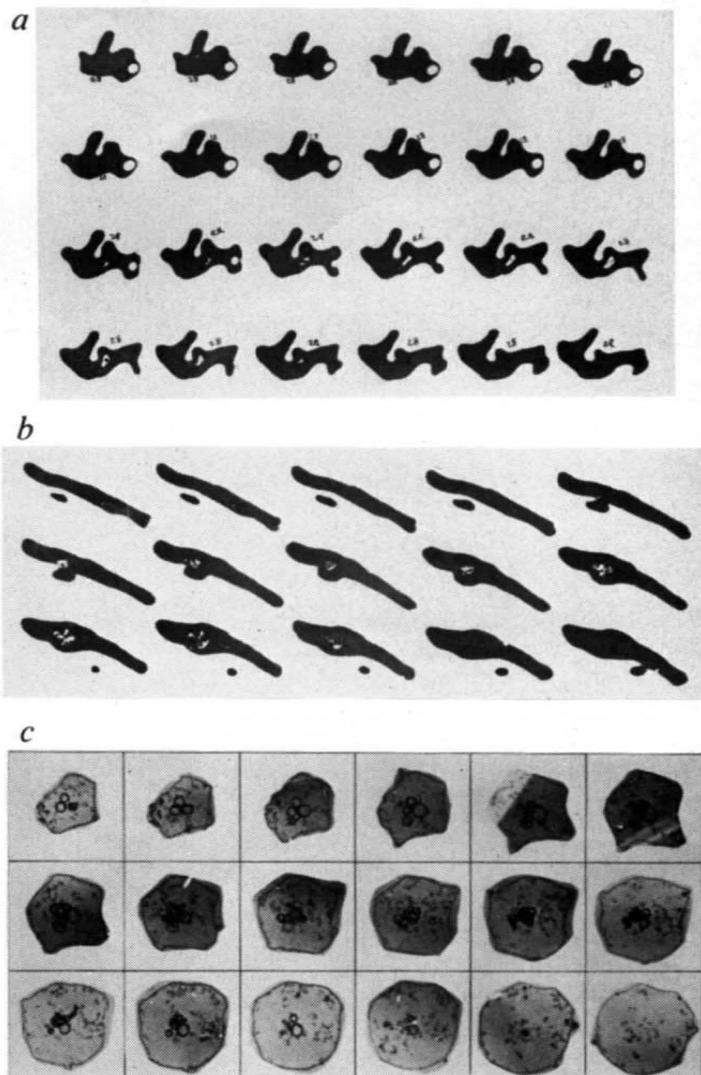
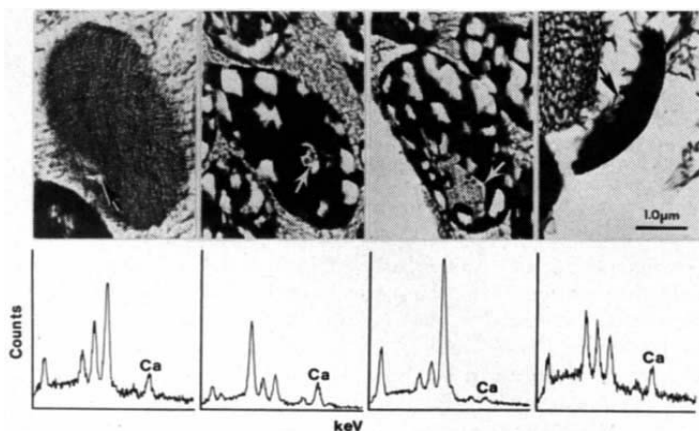


Fig. 2 Top, frozen dried cryosections of the dense ISC-rich fraction of SS cells (see Fig. 4 legend), showing four typical examples of Ca^{2+} -containing vacuoles. The vacuoles are indicated by arrows, and the spectra obtained when the electron beam was focused on the content of the vacuoles are shown beneath each micrograph. The holes in the two red blood cells in the centre are a result of ice crystal damage typically observed in the deeper part of the specimens. Analysis of the walls of the holes gave phosphorus concentrations similar to those of the adjacent cytoplasm, whereas the walls of the calcium-containing vacuoles were high in phosphorus, consistent with their being membrane-enclosed. Occasional cells, such as that illustrated in the extreme left-hand panel, were much less electron-opaque than the majority and, while some also showed vacuoles with high Ca content, the ionic composition of their cytoplasm (not shown) suggested that they were partially haemolysed.

Methods. Small droplets (<1 mm diameter) of the red cells were placed on the end of a bamboo splinter and plunged into super-cooled Freon 22. The droplets were sectioned at -130°C in a cryoultramicrotome and the ~100-nm-thick sections were placed on carbon support films and freeze-dried at $<10^{-5}$ torr. The freeze-dried cryosections were carbon-coated and analysed at -100°C on the cold stage of a Philips 400T transmission electron microscope operated at 80 kV and equipped with a 30-mm² Kevex energy dispersive detector connected to a Kevex multichannel analyser and a PDP11/34 computer. The duration of individual analyses was 150–300 s, with a probe current of ~1 nA. The equipment configuration and its operating characteristics have been described elsewhere^{20,21}.



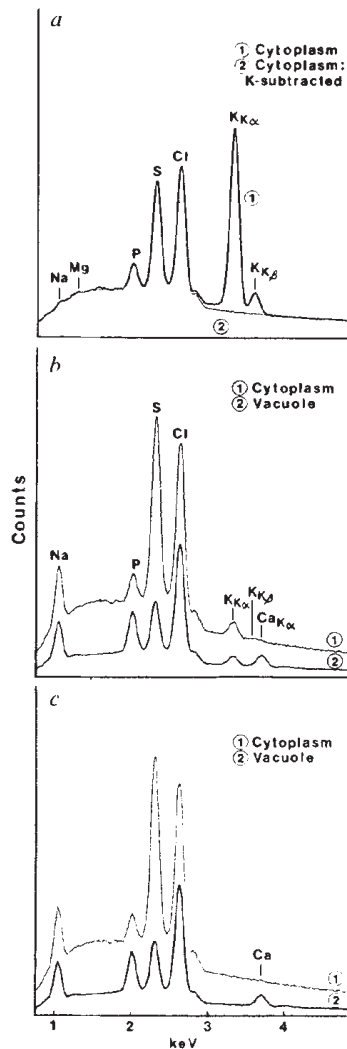


Fig. 3 Averaged X-ray spectra from normal (*a*) and sickle (*b*, *c*) red blood cells. *a*, Spectrum of normal red cells before (1) and after (2) subtraction of the potassium $K\alpha$ and $K\beta$ peaks, showing that the spectrum is flat in the calcium region. *b*, Averaged spectra (15 analyses obtained with focused 100-nm-diameter probes) for the cytoplasm (1) and (15 analyses) with probes focused over the vacuoles (2). $CaK\alpha$ indicates the position of the calcium $K\alpha$ peak. *c*, The same spectrum as *b*, with the potassium $K\alpha$ and $K\beta$ peaks subtracted²⁷ to contrast the high calcium concentration within the vacuoles with the very low concentrations in the cytoplasm.

convoluted shapes. Enclosed vesicles were present in all the SS cells and ghosts. Relative to normal cells, SS vesicles were more numerous and much larger (particularly in the ISCs), showed marked variations in shape, size and numbers per cell, were often multi-lobed and contained electron-dense inclusions.

The compartmentalization of calcium in sickle cells was established directly by electron-probe X-ray microanalysis^{20,21} of cryosections made from the dense, ISC-rich cell fraction. Figure 2 illustrates the X-ray spectra and Ca^{2+} peaks corresponding to representative vesicular images seen in frozen dried sections of ISCs. Figure 3 shows averaged X-ray spectra of normal red blood cells, and the vacuoles and adjacent cytoplasm of ISCs. Highly significant Ca^{2+} peaks were recorded only from the vacuoles that also contained higher concentrations of phosphorus than the adjacent cytoplasm. The P/Ca ratio within the vacuoles varied from 1.5 (the ratio in hydroxyapatite) to 7.6, suggesting that organic phosphates may participate in the formation of precipitates. The calcium content of the vacuoles averaged 35 mmol per kg dry wt. With about 0.3–0.4 kg dry wt per l of cells, and the range of vesicular volumes found in ghosts

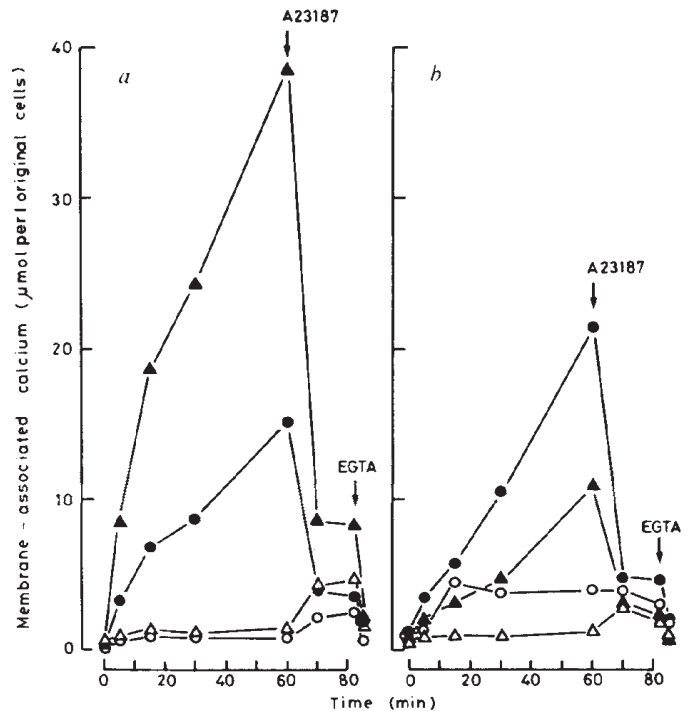


Fig. 4 Calcium uptake by sickle (*a*) and normal (*b*) disrupted red cell membranes in the presence and absence of ATP. Open symbols, ATP-free conditions; solid symbols, ATP added and maintained. ○, ●: Second-lightest cell fraction; △, ▲: densest cell fraction.

Methods. Red cells from normal and sickle-cell anaemic blood were separated on Stractan (arabinogalactan) density gradients²⁸ to obtain the second-lightest fractions (diskocytes free of white cells, platelets and most reticulocytes) and the densest fractions (~70% irreversibly sickled forms (ISCs) from the SS cells). After separation, the cells were washed twice in ice-cold medium A containing (in mM): NaCl, 75; KCl, 75; EGTA 0.1; Tris-Cl pH 7.5, 10, and lysed in 50 vol. ice-cold medium containing 2.5 mM K-HEPES pH 7.6 and 1 mM $MgCl_2$. These conditions are known to completely prevent spontaneous inside-out vesicle formation²². The membranes were centrifuged at 17,000 r.p.m. for 15 min in the cold, and the pellets were frozen and thawed once to ensure complete cell lysis. Each pellet was suspended in 1 ml ice-cold buffer containing 10 mM KCl, 50 mM Na-HEPES pH 7.5, 1 mM $MgCl_2$, 40 μM $^{45}CaCl_2$ and 3 μM calmodulin, and divided in half to receive either 1 mM Na-ATP with 5 mM creatine phosphate and 50 U ml^{-1} creatine phosphokinase (to maintain ATP), or 50 U ml^{-1} hexokinase and 25 mM glucose (to secure complete ATP depletion). After zero-time samples had been taken in the cold, the suspensions were incubated at 37 °C with stirring. Samples (50 μl) were delivered onto Millipore filters (type HA, 0.45 μm , prewashed with the suspension buffer containing, in addition, 2 mM $^{40}CaCl_2$) on a vacuum manifold, washed twice with the same ^{40}Ca -containing buffer, and the filters dissolved in scintillation fluid in counting vials. At the times indicated, ionophore A23187, at a moderately high concentration equivalent to 50 μmol per l of original cells, and subsequently 2 mM Tris-EGTA, were added to the incubation mixtures.

(see Fig. 1 legend), the measured intravesicular calcium concentration can easily account for mean total cell Ca^{2+} values in the range observed^{10,12}, that is, 40–100 μmol per l of cells.

If endocytic inside-out vesicles are to account for the observed calcium accumulation by SS cells during sickling, they must have ATP-dependent Ca^{2+} pumps capable of pumping Ca^{2+} inward, in competition with the Ca^{2+} -extrusion pumps of the outer cell membrane. The ATP-dependent, Ca^{2+} -accumulating capacity of any vesicles that might have resisted the procedures applied to disrupt the outer cell membrane, was investigated by the protocol described in Fig. 4 legend. The results of four similar experiments (see Fig. 4) demonstrate that disrupted

membranes from both SS and normal red cells are capable of substantial ATP-dependent Ca^{2+} accumulation in the presence of non-limiting concentrations of Ca^{2+} -pump substrates and cofactors. That accumulation of Ca^{2+} was uphill was demonstrated by the collapse of the Ca^{2+} gradient on addition of the calcium ionophore A23187 before the addition of excess Ca^{2+} chelator. In all instances, ATP-dependent Ca^{2+} uptake was maximal at the start of the incubation, without noticeable delay; this argues against uptake by newly formed inside-out vesicles as, even under the most favourable conditions for spontaneous endocytosis, more than 6–15 min of incubation at 37 °C are required to produce sealed inside-out vesicles²². Therefore, the observed uptake must represent the Ca^{2+} -accumulating capacity of those endocytic inside-out vesicles that either resisted lysis (possibly the same as Palek's ghost-associated ^{45}Ca fraction⁴), or which re-sealed spontaneously after lysis.

The results presented here explain the paradox of the silent calcium in SS cells and circumscribe the possible role of calcium in the generation of ISCs. Cytoplasmic Ca^{2+} concentrations must be increased transiently, at least, to lead to accumulation within endocytic inside-out vesicles. The possibility remains¹ that in some cells such Ca^{2+} transients could briefly activate K^{+} channels and initiate the process of dehydration (thereby further promoting the sickling process), eventually resulting in ISC formation. Recent work lends some support to this possibility²³.

The endocytic inside-out vesicles present in red cells could record cumulatively any episodes of increased Ca^{2+} permeability as an increase in the calcium content of the cells. In view of the evidence for endocytic inside-out vesicles capable of Ca^{2+} accumulation in normal red cells (Fig. 4), the absence of a measurable pool of ionophore-mobilizable Ca^{2+} in these cells¹⁰ argues against their having periods of substantially increased Ca^{2+} permeability, such as those proposed to occur with normal red cells during microcirculatory shear stress²⁴. A normal spleen, however, might 'pinch-off' Ca^{2+} -loaded vesicles from traversing red cells¹⁹, thus erasing such a record, whereas the functional asplenia of sickle cell anaemia patients²⁵ could account for the persistence of such vesicles.

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Reorganization of α -fodrin induced by stimulation in secretory cells

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Spectrin is an ubiquitous protein composed of heterodimers with α and β subunits. It was first described in erythrocyte cell membranes (see ref. 1 for review) and subsequently in brain, intestinal brush borders, kidney, liver and adrenals^{2–4}. Brain spectrin (fodrin) α -subunit, responsible for actin binding, has a relative molecular mass (M_r) of 240,000, whereas the β -subunit, involved in membrane attachment, has an M_r of 235,000 (refs 1, 3, 9–13). The membrane of secretory granules from adrenal chromaffin cells increases the viscosity of F-actin solution^{6,14}, and spectrin-like protein is associated with storage granule and plasma membranes⁵. Here, we report the localization of fodrin in secretory cells using monospecific antibodies against the α -subunit of fodrin using indirect immunofluorescence. We find that the α -subunit forms an intensely stained continuous ring in the subplasmalemmal region of resting chromaffin cells. On stimulation of the cell with nicotine, high potassium or ionophores in the presence of calcium, fodrin forms patches. This aggregation is inhibited by trifluoperazine, hence the entrance of calcium into cells following cell depolarization seems to be the calmodulin-dependent stimulus initiating patch formation.

The chromaffin cell of the adrenal medulla is often used as a model to study the secretory process in neuroendocrine cells^{15,16}. The recent development of primary cultures of adult chromaffin cells isolated from bovine adrenal glands^{17–20} provides a system that can be used to probe the cellular mechanism underlying secretion.

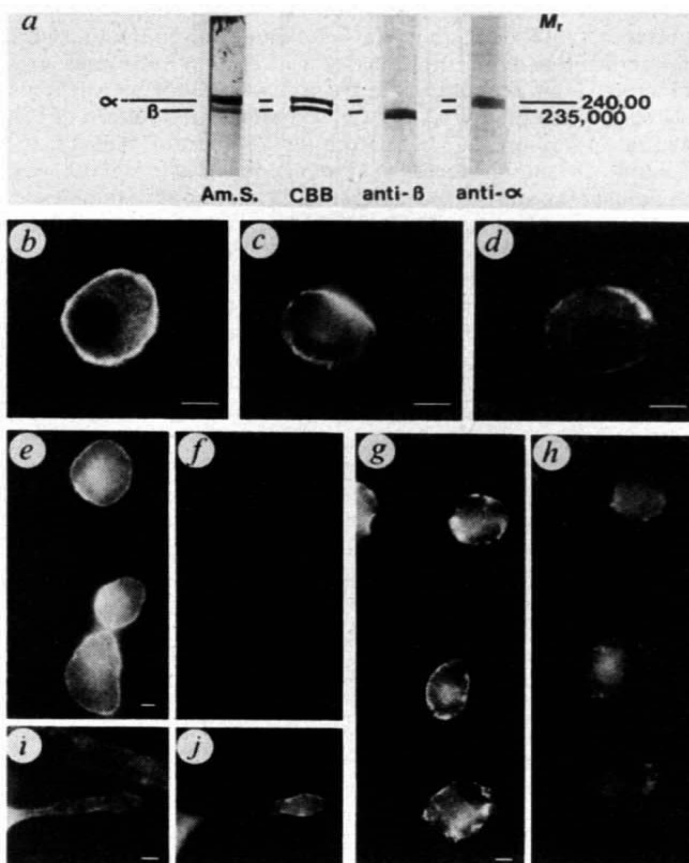
To examine fodrin localization in cultured chromaffin cells we raised antibodies against the α - and β -subunits of bovine brain fodrin. Using immunoaffinity-purified antibody to bovine brain membrane β -fodrin no staining of chromaffin cells was observed, indicating that brain β -fodrin is immunologically distinct from that of paraneuronal cells (Fig. 1a). We confirmed these observations by immunoblotting experiments on chromaffin cell cytoskeletal proteins (data not shown). In contrast, immunoaffinity-purified antibody to the α -subunit of bovine brain stained the chromaffin cell periphery as an intense fluorescent ring (Fig. 1b). The α -fodrin antigens were apparently not accessible to the cell surface because no antibody staining occurred unless the cells were first permeabilized with Triton X-100. Preimmune serum or antiserum absorbed with fodrin did not decorate chromaffin cells. Thus, we conclude that α -fodrin antigens are concentrated in the subplasmalemmal region of cultured chromaffin cells.

In 85 ± 5% of cells from four different primary cultures, α -fodrin was visualized as a continuous ring. In the remaining 15% a different staining pattern was observed; α -fodrin antigens still occurred in the subplasmalemmal region but were aggregated in some places, producing a discontinuous labelling, suggesting that α -fodrin organization is related to secretory activity. Therefore, we stimulated cultured chromaffin cells with nicotine and then stained for α -fodrin antigens. In these experiments, antibody against dopamine β -hydroxylase (DBH), a marker for the inner surface of granule membranes, was also added during the stimulation period. This marker is known to be inserted into the plasma membrane during exocytosis²¹ (Fig. 1g, h). Cells were examined, counted and classified as either showing a continuous subplasmalemmal fodrin ring or discontinuous labelling with patches. We found a close relationship between the number of cells displaying patchy fodrin distribution and

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Fig. 1 Immunofluorescence localization of α -fodrin in cultured chromaffin cells. **a**, Characterization of anti- α -fodrin and anti- β -fodrin antibodies. Bovine brain fodrin α - and β -subunits were separated by polyacrylamide slab gel electrophoresis and stained with Coomassie brilliant blue (lane CBB). After transfer to a nitrocellulose sheet, one lane was stained with Amido black (lane Am.S.), and the other two reacted with either anti- α -fodrin (anti- α) or anti- β -fodrin (anti- β) monospecific immunoaffinity-purified antibody. Bound immunoglobulins were then detected with goat peroxidase-conjugated anti-rabbit immunoglobulins (Nordic Immunologicals) and chloro-2-naphthol^{30,31}. No cross-reaction of anti- α -fodrin with the β -subunit or anti- β -fodrin with the α -subunit was observed. **b-d**, Localization of α -fodrin antigens in resting and stimulated chromaffin cells. Chromaffin cells in culture were incubated for 10 min at 22°C with no secretagogue (**b**) or with 56 mM K⁺ (**c**) or 25 μ M X537A (**d**) in the presence of 2.2 mM Ca²⁺. Cells were then processed for α -fodrin localization using immunofluorescence microscopy. **e-j**, simultaneous localization of α -fodrin antigens (**e**, **g**, **i**) and exocytotic sites (**f**, **h**, **j**) in chromaffin cells in culture stimulated in the absence (pair **e**, **f**) or presence (pairs **g**, **h**; **i**, **j**) of external calcium. Antiserum to DBH was used as a marker for the inner surface of granule membrane as it is transiently becoming accessible during exocytosis. Chromaffin cells in culture were washed and incubated for 10 min at 22°C in 2.2 mM Ca²⁺-Locke's solution containing specific (ref. 6) rat anti DBH antiserum (dilution 1:100) with 10 μ M nicotine (pair **g**, **h**) or 25 μ M X537A (pair **i**, **j**); **e**, **f**, incubation performed in Ca²⁺-free (5 mM EGTA) Locke's solution in the presence of 10 μ M nicotine. Cells were then processed for immunofluorescence microscopy. Scale bars, 10 μ m.

Methods. Anti-fodrin antibodies: Antibodies against fodrin purified from bovine brain membranes³² were raised in rabbits. Brain fodrin was eluted from SDS electrophoresis³³ and injected into rabbits with Freund's complete adjuvant and 3 weeks later with Freund's incomplete adjuvant. To purify monospecific anti- α - and anti- β -fodrin antibodies, purified bovine brain fodrin was separated on 4-8% SDS/polyacrylamide slab gels and transferred to nitrocellulose sheet (30 V; 320 mA; 15 h; see ref. 34). After staining the proteins with Ponceau red, the strip corresponding to each fodrin subunit was cut out and incubated for 60 min at 37°C in a solution of 3% bovine serum albumin in phosphate-buffered saline (PBS). Nitrocellulose strips were then incubated for 120 min at 20°C in anti-fodrin antisera diluted 10-fold with PBS. After extensive washings with PBS, bound immunoglobulins were eluted with 0.2 M glycine-HCl (pH 2.8) for 4-5 min³⁵, and the eluate immediately neutralized with 1 M NaOH. The solution was then dialysed overnight against 0.2 M borate buffer (pH 7.8), analysed at 280 nm, divided into aliquots and stored at -70°C. Chromaffin cells in culture: Chromaffin cells were isolated from fresh bovine adrenal glands by retrograde perfusion with collagenase and purification on self-generating Percoll gradients^{17,31}. Cells were suspended at a concentration of 10⁵ cells ml⁻¹ of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum containing cytosine arabinoside (3 μ g ml⁻¹), streptomycin (50 μ g ml⁻¹) and penicillin (50 U ml⁻¹). Cells were seeded on rat-tail collagen-coated glass coverslips, which were placed into Falcon Petri dishes (35 mm diameter). Cells were cultured at 37°C in an H₂O-saturated atmosphere of 95% air/5% CO₂ and used within 7 days after plating. Immunofluorescence microscopy: After stimulation (as indicated) cells were rapidly (1 min) rinsed in PBS, pH 7.4, and then fixed with 4% formaldehyde in PBS and permeabilized with acetone³⁶. After a further wash, cells were treated with normal swine and goat immunoglobulins (2 mg ml⁻¹), then incubated with monospecific anti- α -fodrin immunoglobulins (1-2 μ g on each coverslip) overnight at 4°C. Finally, antibodies were detected with swine fluorescein-conjugated anti-rabbit immunoglobulins (Nordic Immunologicals; diluted 1:50 in PBS) and, when necessary, with goat rhodamine-conjugated anti-rat immunoglobulins (Nordic Immunologicals; diluted 1:60 in PBS). Coverslips were mounted in 1:1 mixture of 87.5% glycerol and PBS containing 50 mM Tris, 20 mM dithiothreitol at pH 8.1. Preparations were examined with a Zeiss Universal microscope using epi-illumination and a Zeiss Planapo 63 $\times$ /1.4 oil immersion objective, with 450-490-nm and 515-560-nm filters for fluorescein and rhodamine fluorescence, respectively.



the external nicotine concentration (Fig. 2). At 10 μ M nicotine, $\sim 70 \pm 10\%$ of chromaffin cells showed a redistribution of fodrin antigens. There is a linear relationship between α -fodrin reorganization and catecholamines released at a given nicotine concentration (correlation coefficient 0.93, data not shown). Patches of α -fodrin antigens formed in 80% of cells 10 min and 94% of cells 25 min after nicotine stimulation, demonstrating the time-dependency of fodrin reassembly (Fig. 3).

To examine further the aggregation phenomenon of α -fodrin antigens, we show that 56 mM K⁺ also induces reorganization of α -fodrin in the subplasmalemmal space similar to that provoked by nicotine (Fig. 1c). In addition, the formation of patches induced by high potassium and nicotine proceeded with the same time-course (Fig. 3), but fewer cells (55-60%) had K⁺-induced patches. Note that even at relatively high depolarizing K⁺ concentrations, catecholamine secretion is significantly less than with nicotine or acetylcholine²². Chromaffin cells stimulated with nicotine were also examined 60 min after stimulation, when α -fodrin labelling had returned to the level in resting cells even though anti-DBH staining of cell membranes was still visible (data not shown).

Thus, nicotine and high potassium produce similar effects on fodrin organization in secretory cells. Because the chromaffin cell requires external calcium in addition to a stimulus to secrete catecholamines and peptides, we next examined the effect of external calcium on fodrin movements on cells stimulated either with nicotine or high potassium. In the absence of external

calcium ($<10^{-8}$ M), catecholamine secretion was blocked and the alteration of α -fodrin organization was no longer observed (Figs 1e, f; 2). The Ca²⁺-dependence of fodrin reorganization was also examined by incubating cells in the presence of X537A, an ionophore provoking catecholamine release in the presence of calcium. In these experiments, incubation of cells with 25 μ M X537A in the absence of calcium did not result in modification of fodrin localization, whereas in the presence of calcium there was a drastic rearrangement of fodrin (Fig. 1d, i). Treatment of cells with the more specific Ca²⁺ ionophore A23187 gave identical results (data not shown). These data indicate that the main stimulus activating α -fodrin movements results from the rise of intracellular calcium concentration following cell depolarization²³.

The α -subunit of brain fodrin is known to bind calmodulin²⁴. Although α -fodrin from the adrenal medulla has not yet been shown to be calmodulin dependent, we tested the effect of trifluoperazine, a calmodulin antagonist, on α -fodrin organization in secretory cells. Nicotine-induced rearrangement of fodrin was abolished totally in 5×10^{-6} M TFP (Table 1). This concentration also inhibited 90% of the catecholamine release (ref. 25 and V. Ceña and D.A., unpublished results).

Because there is an exocytotic stage of secretion in chromaffin cells, it is possible that fodrin redistribution is the passive consequence of secretory vesicle fusion with the plasma membrane. Stimulation is followed by the addition of much of the vesicle membrane to the plasma membrane; therefore, some

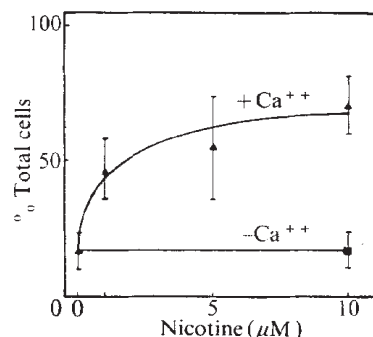


Fig. 2 Effect of nicotine concentration on the number of chromaffin cells showing discontinuous α -fodrin staining. Chromaffin cells in culture were washed with 2.2 mM Ca^{2+} or Ca^{2+} -free (5 mM EGTA) Locke's solution and stimulated for 10 min at 22 °C with nicotine as indicated. Cells were then rapidly washed, fixed and processed for α -fodrin localization as described in Fig. 1 legend. For each nicotine concentration, 150 cells were examined and classified. Experiments were repeated three times on three different cultures of chromaffin cells. Each value is the mean $\pm$ s.d.

clearing of plasma membrane proteins would be expected. In separate experiments (O. K. Langley, D.P. and D.A., unpublished results), resting and nicotine-stimulated chromaffin cells were labelled with anti-D2 antibody, D2 being a glycoprotein belonging to the family of cell-adhesion molecules and found on the surface of all chromaffin cells²⁶. No alteration of D2 localization was found. In addition, examination of the paired cells in Fig. 1g, h, did not show any obvious correlation between fodrin patches and exocytotic sites; the latter has been visualized with the anti-DBH antiserum. Therefore, the possibility that fodrin movements result only from vesicle membrane integration into cell membrane is highly unlikely.

The redistribution of α -fodrin in the subplasmalemmal region of cultured secretory cells after stimulation of the cell-surface receptor suggests that the subplasmalemmal cytoskeletal organization is affected by interactions between surface receptors, internal calcium concentration, calmodulin and environmental stimuli. The organization of actin in stimulated cultured cells was also examined, but changes in the actin distribution were much more difficult to evaluate because its distribution¹⁸ in resting cells is variable (that is, it is either localized as fibres or dots, diffuse in the cytoplasm or underlying the cell membrane).

The present study is the first demonstration that fodrin is redistributed when secretory cells are stimulated externally. Fodrin-related proteins have been localized previously in the cortical cytoplasm of many cells and found to redistribute during ligand-induced aggregation of receptors in fibroblasts and during capping on B and T lymphocytes^{27,28}. 'Patching' has been described as a segregation of plasma membrane-associated macromolecules to certain domains of the membrane. Hence, fodrin seems to mediate surface receptor interactions with the cytoskeleton, and may act as a specific linker of certain membrane-associated proteins to the filamentous cytoskeleton.

Table 1 Effect of trifluoperazine (TFP) on α -fodrin distribution in resting and stimulated chromaffin cells in culture

Experimental conditions (in 2.2 mM Ca^{2+} - containing Locke's solution)	No. of cells showing α -fodrin rearrangement (%)
Control	5-10
Nicotine 10 μM , 10 min	80-90
TFP 5.10^{-7} M, 20 min + nicotine 10 μM , 10 min	60-65
TFP 5.10^{-6} M, 20 min + nicotine 10 μM , 10 min	5-10
TFP 5.10^{-6} M, 20 min	5-10

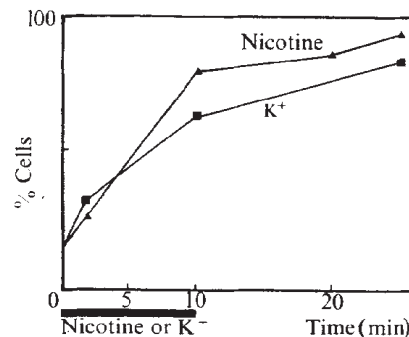


Fig. 3 Kinetics of α -fodrin patch formation in chromaffin cells. At indicated times after stimulation either with 10 μM nicotine or 56 mM K^{+} at 22 °C in the presence of 2.2 mM Ca^{2+} , cells were fixed, stained for α -fodrin (see Fig. 1 legend) and counted. Stimulation with secretagogue was for a maximum of 10 min and then cells were washed with Locke's solution. Each value is the mean of two experiments.

In addition, fodrin movements may also be involved with granule movement. The subcortical cytoplasm of eukaryotic cells where fodrin seems to be concentrated is characterized by a dense fibrillar array of interacting filaments and associated proteins. In resting cells the fodrin fluorescence ring probably reflects this subplasmalemmal dense network, which, when gelled, could act as a physical barrier preventing granules from moving towards the cell membrane and undergoing exocytosis. In stimulated cells, fodrin movements may cause the appearance of free spaces in the continuous ring resulting from a calcium-dependent solation and thereby allow granules to move. This solation process seems to be calmodulin dependent because trifluoperazine blocks both secretion²⁵ and fodrin movements. It is of interest to note that trifluoperazine-treated cells have been shown to contain an increased number of granules within 200 nm of the plasma membrane²⁹.

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Production of human α -interferon in silkworm using a baculovirus vector

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Microorganisms are generally used for mass production of foreign gene products, but multicellular organisms such as plants have been proposed as an economical alternative. The silkworm may be useful in this context as it can be cultured easily and at low cost. We have therefore developed a virus vector to introduce foreign genes, for example, the gene for human α -interferon (IFN- α), into silkworms. We used the baculovirus *Bombyx mori* nuclear polyhedrosis virus (BmNPV) which has a large (>100 kilobases, kb) double-stranded circular DNA genome within its rod-shaped capsid. Baculoviruses have been used previously as vectors for expression of β -interferon and β -galactosidase in established cell lines^{1,2}. Although BmNPV has not been used previously as an expression vector, it has an advantage over the baculovirus *Autographa californica* NPV in that it has a narrower host range and will not grow in wild insect pests in the field. In the present study, the polyhedrin gene encoding the major inclusion body protein of BmNPV was identified by hybridization with complementary DNA and cloned in a plasmid. For insertion of foreign genes, we constructed a recombinant plasmid carrying a polylinker linked to the promoter of the polyhedrin gene, and inserted the IFN- α gene into this plasmid. The resulting plasmid and the BmNPV genomic DNA were co-transfected into BM-N cells, and stable recombinant viruses isolated by plaque assay on BM-N cells. The recombinant virus replicated in silkworm larvae, which synthesized as much as 5×10^7 units ($\sim 50 \mu\text{g}$) of interferon in their haemolymph.

We used BmNPV T3 isolate, which has been plaque-purified on BM-N cells³. To identify the polyhedrin gene on the viral genome of the BmNPV T3 isolate, cDNA made from messenger RNA isolated from infected fat bodies was used as a probe. In *in vitro* translation experiments in reticulocyte lysates, over 90% of the total mRNA obtained from these fat bodies at a late stage of infection was found to encode the polyhedral protein. The labelled cDNA synthesized by reverse transcriptase was hybridized to viral DNA fragments separated on an agarose gel after cleavage with restriction endonucleases. An *EcoRI* 10.5-kb fragment or a *HindIII* 3.9-kb fragment hybridized specifically to the probe. The *EcoRI* 10.5-kb fragment was cloned into pBR322 at the *EcoRI* site. Figure 1 shows a physical map of the resulting plasmid (pBmE36). Further hybridization experiments revealed that a 1.8-kb *HpaI*-*HindIII* fragment in pBmE36 hybridized best with the probe and contained the gene for the polyhedral protein gene.

The nucleotide sequence of 1,763 bases from the *HpaI*-*HindIII* fragment was determined by the dideoxy chain termination procedure^{4,5}; the inferred amino-acid sequence was com-

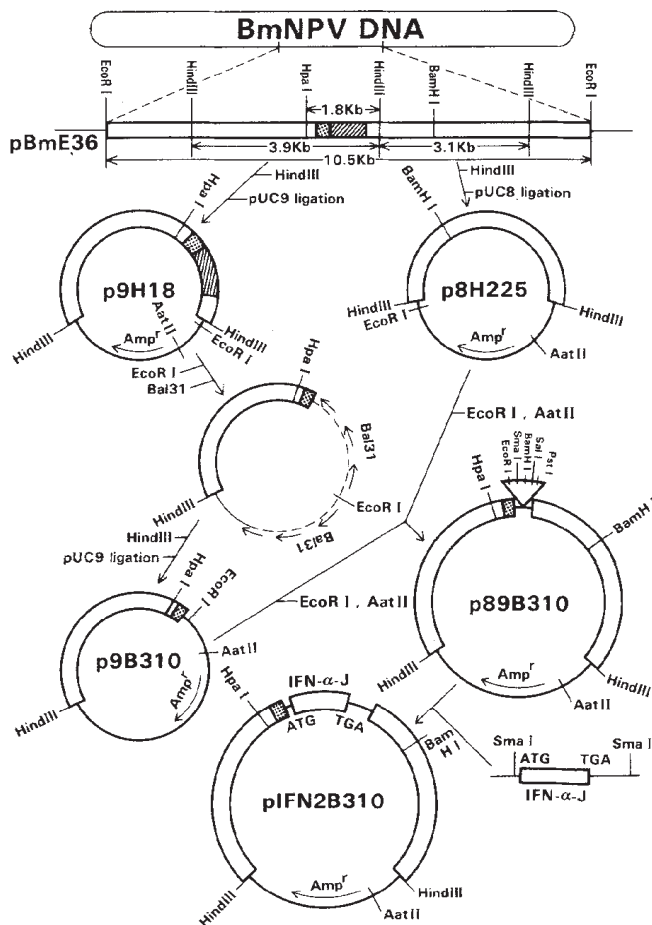


Fig. 1 Construction of vectors. The plasmid pBmE36 was cleaved with *HindIII* to obtain the 3.9-kb *HindIII* fragment containing the entire polyhedrin gene (represented by hatched blocks). This fragment was inserted into the *HindIII* site of pUC9. The resulting plasmid, p9H18 (50 μg), was cleaved with *EcoRI*, then incubated at 23 °C for 10 min with 50 U of *Bal31* exonuclease. Samples (100 μl) were removed at 2-min intervals and the reaction stopped by adding 0.2 vol. 0.5 M EDTA. The samples were extracted with phenol and then chloroform, and precipitated with ethanol. The truncated p9H18 fragments were digested with *HindIII*, and 2.7-kb *HindIII* blunt-end fragments were isolated from the agarose gel. This fragment was ligated to the *HindIII*-*SmaI* site of pUC9 (p9B310). Sequencing of p9B310 revealed that the polyhedrin gene was digested as far as position -18 upstream of the first codon, ATG (see Fig. 2). A 3.1-kb *HindIII* fragment of pBmE36 was ligated to pUC8 and the resulting plasmid, p8H225, cleaved by *EcoRI* and *AatII* to obtain the 3.5-kb fragment containing 3.1 kb of DNA downstream of the polyhedrin gene; this fragment was then ligated to the 4.7-kb *EcoRI*-*AatII* fragment of p9B310. The resulting hybrid plasmid, p89B310, contains a polylinker (*EcoRI*, *BamHI*, *SmaI*, *Sall* and *PstI* sites) just downstream of the promoter (represented by dotted blocks) of the polyhedrin gene. An IFN- α -J gene, which has *SmaI* sites just before the ATG start signal (... CCCGGGATG...), was ligated to p89B310 and cleaved by *SmaI* to construct pIFN2B310 (Fig. 2). In this plasmid, the IFN- α -J gene was oriented in the same 5' to 3' direction as the polyhedrin gene. The IFN- α -J gene used for this construct contained the signal peptide sequences, the coding sequences of mature IFN- α -J and the 3'-noncoding sequences (~ 670 bases from the stop signal, TAG) of interferon, including the signal for polyadenylation.

pared with that of the polyhedrin protein of BmNPV⁶. The starting codon, ATG, of the polyhedrin protein was found 580 bases downstream from the *HpaI* site and the stop codon TAA was found 448 bases upstream from the *HindIII* site. More than 85% of the deduced amino-acid sequence was homologous to that reported by others for *Autographa californica* NPV (AcNPV)^{1,2}. The differences show the degree of evolutionary

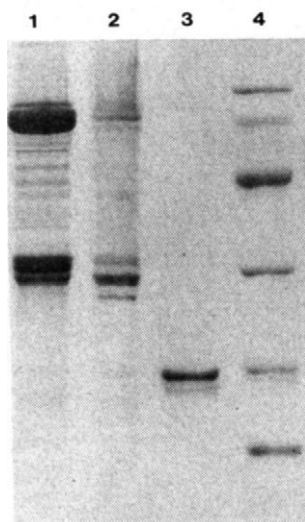


Fig. 4 Purification of the interferon produced in the silkworm. Lane 1, 5 μ l of uninfected haemolymph; lane 2, 5 μ l of BmIFN2B310-infected haemolymph; lane 3, 5 μ l (20 μ g protein) of the fraction eluted from the affinity chromatography column; lane 4, 5 μ g of each relative molecular mass (M_r) marker (phosphorylase *b*, M_r 94,000 (94K); albumin, 67K; ovalbumin, 43K; carbonic anhydrase, 30K; trypsin inhibitor, 20.1K; α -lactalbumin, 14.4K).

Methods. About 10 ml of haemolymph from 15 silkworm larvae infected with BmIFN2B310 was collected as described in the text. The haemolymph was mixed with an equal volume of fresh medium for BM-N cells and centrifuged at 12,000 r.p.m. for 5 min. The supernatant was applied to an affinity Sepharose 4B column with monoclonal antibody against human IFN- α and washed several times with a solvent containing 0.2 M sodium acetate (pH 7.3), 0.5 M NaCl and 20% glycerol. The interferon was eluted from the column with a solvent containing 0.1 M citric acid (pH 2.2), 0.5 M NaCl and 20% glycerol, and concentrated ~10-fold. Samples were analysed by SDS-polyacrylamide gel electrophoresis on a 10% gel. The gel was stained with Coomassie brilliant blue R.

without degradation by endogenous enzymes and can be purified without apparent loss of activity.

The present study represents the first report of a high level of synthesis of a foreign gene product in a living animal. The silkworm has been improved for mass culture for several thousand years, and it is now possible to maintain unlimited amounts of silkworm under the control of computerized machines. The system described here, which uses these insects, thus offers an exciting alternative for mass production of foreign gene products.

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Retrovirus-induced *de novo* methylation of flanking host sequences correlates with gene inactivity

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The pattern of DNA methylation changes during development of eukaryotes, and hypomethylation frequently correlates with gene expression (for reviews see refs 1-4). A causal relationship between hypermethylation and gene inactivity has been established for retroviral genomes which are methylated *de novo* when inserted into the germ line of mice (ref. 5; for review, see ref. 6). The mutual interaction of the provirus with the host genome can influence virus expression⁷ and can result in inactivation of the host gene by insertional mutagenesis⁸. We report here that the insertion of a provirus can change the methylation pattern of the host DNA. Sequences flanking the provirus become methylated *de novo* within 1 kilobase (kb) of the integration site. In Mov-13 mice, which carry a lethal mutation of the $\alpha 1(I)$ collagen gene^{9,10}, *de novo* methylation of host DNA is associated with a change in chromatin conformation¹¹. This suggests that virus-induced DNA methylation can alter DNA-protein interactions and thereby interfere with correct gene activation during embryonic development.

The Mov substrains of mice, each carrying a single and highly methylated Moloney murine leukaemia virus (M-MuLV) genome at a distinct mendelian locus⁷, were used to study the interaction of a provirus with its flanking host sequences. DNAs from parental control mice and from Mov mice were compared to investigate whether insertion of virus affects the methylation state of its flanking sequences. Methylation patterns were analysed by digesting DNAs from different developmental stages with methylation-sensitive restriction enzymes, followed by hybridization with probes representing the viral preintegration site. Here we report the results obtained with Mov-7, Mov-9 and Mov-13 mice.

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was at a maximum (4×10^7 U ml⁻¹) at day 4. The highest recorded level of human IFN- α produced in a eukaryotic system is 4×10^5 U ml⁻¹ (for mouse cells with a bovine papillomavirus vector⁸).

The haemolymph from the infected silkworm was affinity-purified by column chromatography, then analysed by SDS-polyacrylamide gel electrophoresis. From 10 ml of infected haemolymph (250 mg protein) collected from 15 larvae, 0.3 mg of interferon was eluted. The specific activity (U mg⁻¹ protein) increased from 2.1×10^5 to 1.0×10^8 ; the yield was higher than 50%. The eluted sample, separated on gels, was stained with Coomassie brilliant blue, which revealed a major band of relative molecular mass 19,500 (Fig. 4). The protein fraction corresponding to this band was extracted from the gel and showed interferon activity. An immune blot analysis indicated that labelled antibody to human IFN- α reacted exclusively with the major band. N-terminal amino-acid sequence analysis (11 amino-acid residues) of the major fraction established its identity to human IFN- α . These findings show that there is no difference between interferon produced in silkworm and that made in mammals, hence the signal peptide sequence can be removed in the same manner as in mammalian cells. Purification of IFN- α made in silkworm is just as easy as for that produced in mammalian cells.

The circulatory system in the silkworm is 'open', in contrast to the 'closed' system of vertebrates. About 30% of the total volume of the silkworm larva consists of the body cavity filled with haemolymph. The haemolymph also represents a storage tissue, for example, the major proteins of the silkworm *Hyalophora cecropia*, called storage proteins, accumulate in the haemolymph at the late larval stage⁹. The interferon produced is probably deposited in the haemolymph in a similar way

Fig. 1 Methylation of sequences 5' of the integration site of the M-MuLV genome in Mov-7. DNAs were prepared from mature sperm, from pools of embryos and embryonic tissues, and analysed with restriction enzymes as described elsewhere¹⁴. Samples (20 µg) were digested with *Bst*EII (B) and in addition with either *Hha*I (H) or *Hpa*II (Hp), fragments were separated on a 1.4% agarose gel, transferred to nylon membrane and hybridized with nick-translated²⁵ plasmid p7-1-1 which carries the *Pst*I(P)-*Eco*RI(E) fragment (solid bar in map at bottom) from the 5'-flanking region of the Mov-7 M-MuLV genome²⁶. Cleavage products were mapped by comparison with the sizes of marker (M) bands derived from plasmid p7-2, which carries the Mov-7 provirus (open bar in map) within flanking cellular sequences. Digestion of p7-2 with *Bst*EII and *Bst*EII plus *Pst*I generated fragments B₂B₃ and B₂P, respectively, and, in addition, fragments of 8.6 and 4.7 kb, respectively, containing vector sequence. Partial methylation at sites H and Hp in Mov-7 sperm was indicated by the loss of intensity of band B₂B₃ and the appearance of weakly hybridizing bands migrating slightly above band B₂P (see map). The complete digestion of bands B₂B₄ (which were not transferred quantitatively to the nylon membrane), the high intensity of the cleavage products B₂H and B₂Hp, and the absence of other products, indicated lack of methylation at sites H and Hp in the DNAs from 129 mice.

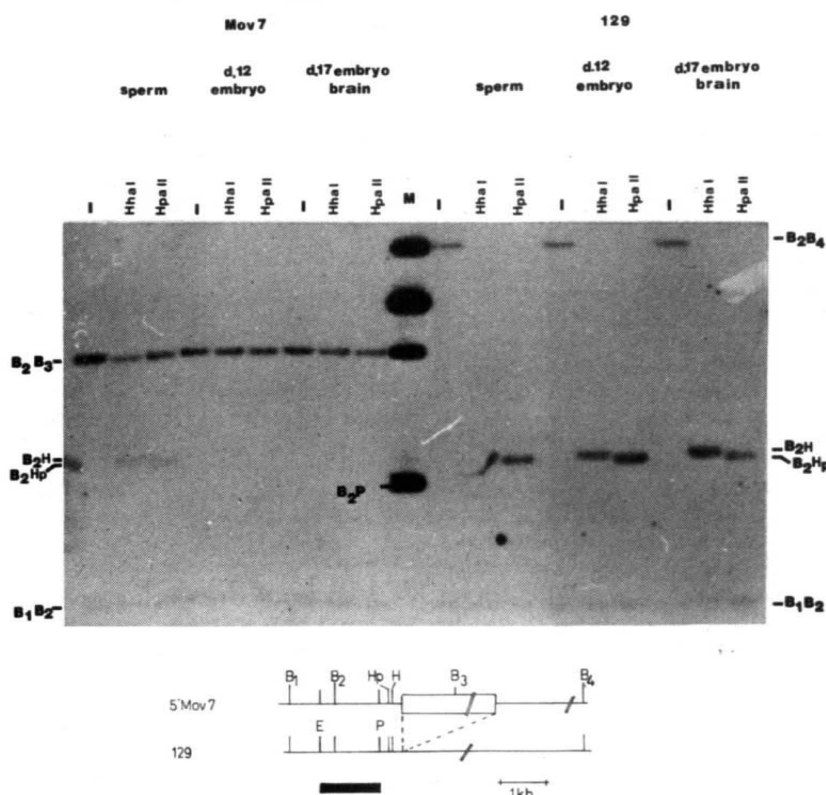


Figure 1 shows *Bst*EII digests of DNA from sperm, and from day 12 and day 17 embryos of the Mov-7 and parental 129 mouse strains, which were hybridized to a probe representing 5'-flanking host sequences. This yielded the virus/cell junction fragment B₂B₃ in Mov-7 and fragment B₂B₄ spanning the integration site in 129 DNA. The junction fragments from day 12 and day 17 embryos of the Mov-7 strain were resistant to cleavage by *Hha*I and *Hpa*II, indicating complete methylation of the sites Hp and H in the 5'-flanking region (see the map in Fig. 1). A slightly lower level of methylation in sperm of Mov-7 mice resulted in digestion of the Mov-7-specific *Bst*EII fragment and generated weakly hybridizing cleavage products. In contrast, both sites were completely unmethylated before virus integration, as demonstrated by the amount and the size of the *Hha*I and *Hpa*II cleavage products in 129 DNA (see Fig. 1 legend).

Similarly to Mov-7, the sequences flanking the provirus in Mov-13 mice were unmethylated before virus integration and became methylated after integration. Figure 2a, b shows the analysis of the 5'-flanking sequences and Fig. 2c that of the 3'-flanking sequence; the DNA bands shown are designated by letters corresponding to the respective restriction enzyme recognition sites indicated on the map. Because Mov-13 mice are heterozygous for the virus insertion⁸, the probes recognize the virus-carrying and the normal allele in these mice and only the normal allele in the C57BL/6 parental strain. Hybridization with probe A (see map in Fig. 2), which identifies sequences 5' of M-MuLV integration site of the Mov-13 strain, revealed identical restriction patterns in *Bst*EII/*Ava*I- (Fig. 2a), *Bst*EII/*Hha*I- (Fig. 2b) and *Bst*EII/*Hpa*II (data not shown)-digested DNAs from sperm of both Mov-13 and C57BL/6 parental mice. The intensity and size of the digestion fragments (see Fig. 2 legend) indicated the absence of methylation at all seven sites within 1 kb of 5' cellular sequences before and after provirus insertion (see Fig. 3). The same patterns as in sperm DNAs were present in the DNAs from day 12 embryos of C57BL/6 and Mov-13 mice (Fig. 2a, b). However, additional bands of higher relative molecular mass in Mov-13 embryo DNA indicated methylation at four sites present within 700 base pairs (bp) 5' of the virus integration site (see Fig. 2 legend). An analysis of a 5' virus/cell

junction fragment, by *Kpn*I digestion of the same DNA, was used to distinguish between the two different chromosomes in the Mov-13 embryos, and localized the *de novo* methylated sites exclusively to the Mov-13 allele (Fig. 3).

The *Bam*HI-*Bgl*II cell virus junction fragment recognized by probe B served to determine the methylation of sequences 3' of the provirus in the Mov-13 allele (Ba-Bg) and in the allele from C57BL/6 parental mice (Ba₁Bg; see Fig. 2c). *Ava*I and *Hpa*II cleaved these fragments completely in sperm DNA from Mov-13 and C57BL/6 mice, generating fragments that indicated non-methylation of the *Ava*I site and of two *Hpa*II sites within 300 bp 3' of the provirus. The same three sites were completely methylated in the Mov-13 allele of day 12 embryos, as the virus/cell junction fragment was nearly resistant to cleavage. In contrast, the normal allele in both Mov-13 and C57BL/6 parental mice was not methylated at two sites, and only partially methylated at the one other site (see Fig. 2 legend; Fig. 3). The single *Hha*I site in this region was analysed similarly by *Sst*I digestion. The methylation at this site in sperm and day 12 embryos was partial in the C57BL/6 parental allele and complete near the Mov-13 provirus (Fig. 3).

The provirus in Mov-9 mice is flanked by cellular sequences which are methylated in day 12 embryos of the 129 parental mice as well as of the Mov-9 strain. Figure 4 shows that five CpG sites within 4 kb of cellular sequences at the 3' end, and one site at the 5' end, are fully methylated in embryonic DNA from the 129 parental strain. Provirus integration at the Mov-9 locus did not change the high level of methylation of the flanking host sequences, except for the *Hha*I site 5' of the provirus, which is partially methylated in sperm of the Mov-9 strain and unmethylated in sperm of the parental 129 strain (Figs 3, 4).

Figure 3 summarizes our results. The cellular sequences at the respective preintegration sites of the Mov-7 and Mov-13 provirus were unmethylated in sperm as well as in somatic cells from day 12 and day 17 embryos and from adult mice of both the 129 and C57BL/6 parental strains. Analysis of somatic cells showed that provirus integration resulted in complete methylation of all tested sites in 1 kb of flanking sequences at both loci. The methylation status of sites that were farther than 1 kb away

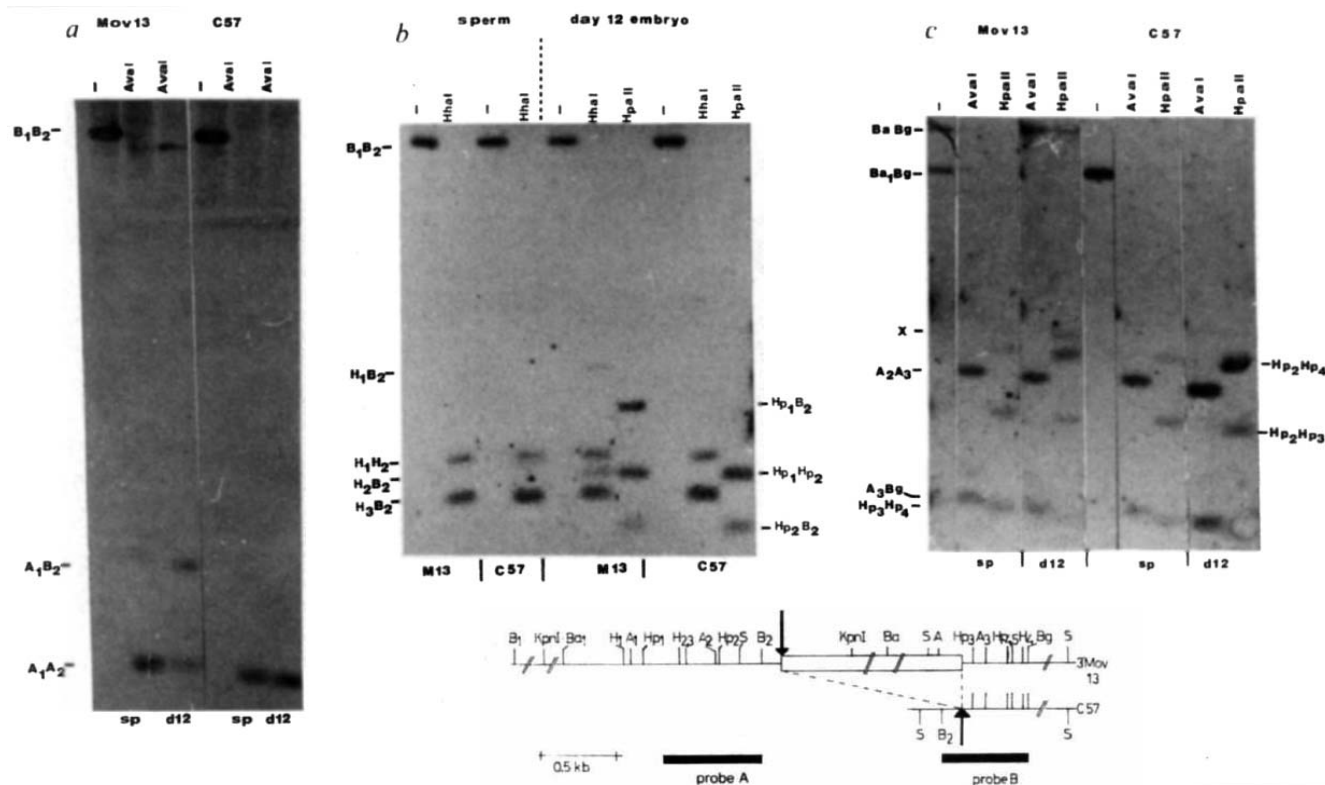


Fig. 2 Methylation of cellular sequences flanking the integration site of the Mov-13 provirus. DNAs from sperm and day 12 embryos of heterozygous Mov-13×C57BL/6 mice⁷ carrying a single M-MuLV provirus (open bar in map) and from uninfected C57BL/6 mice were prepared and analysed as described in Fig. 1 legend. The methylation of cellular sequences 5' of the proviral integration site (vertical arrow) was determined in 20-μg samples of *Bst*EII (B)-digested DNAs with *Ava*I (A) (a), *Hha*I (H) and *Hpa*II (Hp) (b), using probe A (black bar) derived from cloned sequences of the Mov-13 locus¹⁰. The nucleotide sequence and map from this region¹⁰ were used to deduce the recognition sites of the methylation-sensitive enzymes (see map) and cloned subfragments of the Mov-13 locus served as length markers (not shown) to map the cleavage sites of these enzymes in the mouse DNAs. Thus, the lengths of the *Ava*I cleavage products in a indicated the absence of methylation at sites A₁ and A₂ in C57BL/6 chromosomes generating band A₁B₂ and methylation at site A₂ in the Mov-13 allele of day 12 embryos was indicated by band A₁B₂. Band A₂B₂ was too small to be detected in this analysis. Similarly, there was Mov-13-specific methylation at sites H₂, H₃ and Hp₂ but not at sites H₁ and Hp₁, as deduced from bands H₁B₂, H₂B₂ and Hp₁B₂, respectively, which appeared in embryonic DNA (b). Complete digestion of Mov-13 and C57BL/6 DNAs indicated the absence of methylation at all *Hha*I and *Hpa*II sites in sperm (b, *Hpa*II analysis not shown). c, The 3' cellular sequences were analysed with probe B (black bar), a *Bst*EII–*Bgl*III fragment from the Mov-13 locus¹⁰. The *Bam*HI–*Bgl*III virus/cell junction fragment of 2.3 kb (Ba–Bg) and the corresponding cellular fragment of 1.7 kb (Ba₁Bg) were further digested with *Ava*I and *Hpa*II. The sizes of the cleavage products derived from DNAs of Mov-13 and C57BL/6 sperm indicated the absence of methylation at sites A₃, Hp₃ and Hp₄ in both alleles. The virus/cell fragments, generated from cleavage at the unmethylated 3' viral sites A and Hp (see ref. 14) were not detectable due to their small stretch of homology with probe B (see map). In day 12 embryos, a fragment of 710 bp showed partial methylation at site Hp₃ in the C57BL/6 allele, and the resistance of fragment Ba–Bg to secondary digestion indicated methylation at all sites 3' of the provirus. Fragment X was generated in DNA from Mov-13 embryos as a result of partial *Hpa*II cleavage at a site in the 3' enhancer region of the provirus, which becomes preferentially demethylated during mouse development¹⁴. The methylation at site H₄ was determined in a similar manner by *SS*I (S) and *Hha*I digestion using probe B (not shown; see Fig. 3).

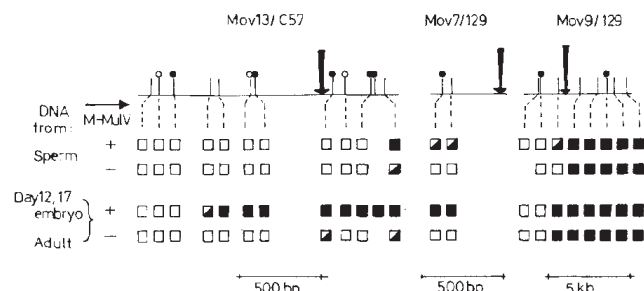


Fig. 3 The methylation at *Hha*I (□), *Ava*I (◐) and *Hpa*II (●) sites around M-MuLV integration sites (vertical arrows) was determined in Mov-7, -9 and -13 mice (+) and in homologous sequences of parental mouse strains (–) as described in the legends of Figs 1, 2 and 4. Filled, half-filled or open boxes indicate ≥80%, 20–80% or <20% methylation, respectively, of the individual sites. The horizontal arrow shows the direction of viral transcription from the 5' to the 3' end. The data for day 17 embryos and adult mice represent liver and brain or brain and kidney, respectively; the analysis of 5' cellular sites from Mov-9 mice is described elsewhere¹⁴.

from the integration site was not affected by provirus integration. The sequences flanking the Mov-13 provirus, which represent the 5' end of the $\alpha 1$ (I) collagen gene¹⁰, were completely unmethylated in sperm, indicating that virus insertion induced *de novo* methylation of unique cellular sequences during embryogenesis. So far, *de novo* methylation has been observed only in repetitive sequences^{12,13} or with proviral or cellular sequences introduced into transgenic mice^{14,21}. The lower level of methylation in sperm than in somatic cells of Mov-13 mice may indicate either germline-specific lack of *de novo* methylation or specific demethylation at these sites during spermatogenesis. The relative hypomethylation of satellite DNA in extraembryonic as opposed to embryonic tissues¹² is consistent with the notion that *de novo* methylation occurs after the blastocyst stage, when these cell lineages have separated.

In contrast to Mov-7 and Mov-13, the provirus in Mov-9 mice has integrated into a hypermethylated region, the methylation status of which was not detectably affected by the insertion. A similar result was found for Mov-2 and Mov-3 (unpublished) and Mov-10 mice¹⁴.

De novo methylation of retroviral sequences introduced into mouse zygotes, early mouse embryos or embryonic carcinoma

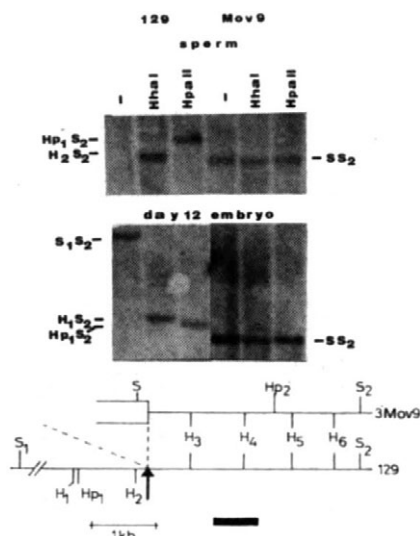


Fig. 4 Methylation of cellular sequences flanking the integration site of the Mov-9 provirus. DNAs from Mov-9 (ref. 7) and uninfected parental 129 mice were prepared and analysed with restriction enzymes as described in Fig. 1, legend, using plasmid p9-1-2, which carries sequences (black bar in map) 3' of the integration site (vertical arrow) of the Mov-9 provirus (open bar; see ref. 26). DNA samples (20 µg) were digested with *Sst*I (S), generating the viral junction fragment SS₂ in Mov-9 DNAs and fragment S₁S₂ which spans the integration site in the 129 alleles. In 3'-flanking sequences the *Hha*I (H) and *Hpa*II (Hp) sites were methylated in Mov-9 mice, as seen by the resistance of band SS₂ to secondary digestion. The gel position of this fragment was used to localize, in 129 mice, the unmethylated sites Hp₁, H₂ or H₁ in sperm and day 12 embryos, respectively (see map).

cells correlates with transcriptional inactivity, whereas the absence of *de novo* methylation in somatic cells correlates with gene expression (refs 15–20; see ref. 3 for review). The present results show that a functional provirus becomes repressed even when integrated into a chromosomal region which, as in Mov-13 mice, has all the characteristics of active gene expression, that is, hypomethylation and the potential to develop an open chromatin configuration¹¹. Thus, the integration of retroviruses may lead to altered chromatin structures that allow *de novo* methylation. The molecular basis for both provirus and host gene suppression may be the maintenance of these structures due to their methylation. Non-retroviral genes which have been introduced into the germ line of mice are often not expressed^{21–24}; it remains to be seen whether this is correlated with similar changes in chromatin structure and methylation pattern as described here for Mov mice.

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A viral enhancer element specifically active in human haematopoietic cells

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One particular class of DNA regulatory elements, the enhancers or activators, can, relatively independently of distance and orientation, dramatically increase the transcriptional activity of homologous and heterologous promoters located in *cis* (see refs 1–3 for reviews, also refs 4–6). Sequence differences between various heterologous enhancers^{7,8} may explain their apparent host-and/or tissue-specific action^{9–15}. Furthermore, differences in the transcriptional control elements may contribute to viral tropism. At least for murine leukaemia virus isolates, thymotropism and leukaemogenicity have been attributed to alterations within the viral long terminal repeat^{15–17}, which harbours their enhancers and other transcriptional control elements. We report here the identification of a viral enhancer element possessing a very restricted tissue range. The enhancer is active in all human cells of the haematopoietic system tested, but not in cells of fibroblast or epithelial origin.

In the recently discovered lymphotropic papovavirus (LPV)¹⁸, progeny production is restricted to continuously growing cells of primate B-lymphocyte origin^{19–21}, and sequence analysis²² shows that the LPV genome has a structure typical of all other known polyomaviruses²³ (Fig. 1). In addition to early and late regions encoding virus-specific proteins, a noncoding region of ~700 base pairs (bp) is situated between the first early and late translational start codons and harbours several sets of repeated sequences. A large set of direct repeats (106 bp) presumably contains the origin of replication (Fig. 1)^{22,23}. Because it is present twice on the cloned LPV DNA, this clone was probably produced from a natural variant carrying two origins of replication; this notion was confirmed experimentally by removing one of the direct repeats, for, despite this deletion, the LPV genome retains its viability (M.P., unpublished). A smaller (63 bp) sequence set lies to the late side of the 106-bp fragment. It is duplicated once flanking the origin proximal to the early region, and is present as a monomer flanking the distal origin (Fig. 1).

Several indications prompted us to search for an enhancer element in this smaller repeat. Its position on the genome is similar to that of all other known papovavirus enhancers^{4,24–28}, with the exception of papillomaviruses²⁹, and a sequence resembling the enhancer core sequence of simian virus 40 (SV40)⁷ and the mouse immunoglobulin constant-region heavy-chain gene (C_μ)¹⁰ is present within the 63-bp repeat. To test for enhancer function, a 234-bp *Hae*III fragment that included the 63-bp repeat (Fig. 1) was cloned into the pA10-CAT2 plasmid⁹,

Table 1 CAT activity in cells of haematopoietic, epithelial and fibroblast origin after transfection with pSV2-CAT, pLPV-5'-CATs or pC μ -5'-CATs DNA

Cell line	Origin	pSV2-CAT	CAT activity pLPV5'-CATs	pC μ 5'-CATs
Haematopoietic cells ^{37,43,44}				
K562	Chronic myeloid leukaemia	++	++	-
HL-60	Human acute promyelocytic leukaemia	(+)	(+)	-
KG-1	Human early myeloblast	+	+++	-
U937	Human myeloid sarcoma	+++	+	-
KM-3	Human non-B, non-T acute lymphoblastic leukaemia	++	++	-
LBL-1	Epstein-Barr Virus (EBV)-transformed cord blood lymphocytes	+++	+	ND
LBL-AK	EBV-transformed cord blood lymphocytes	+++	+	ND
BJAB	Human Burkitt's lymphoma, EBV-negative	+++	++	++
P3HR-1	Human Burkitt's lymphoma, EBV-positive	+++	++	ND
Daudi	Human Burkitt's lymphoma, EBV-positive	+++	+	ND
JM-1	Human acute T-cell lymphoblastic leukaemia	++	++	-
MOLT-4	Human acute T-cell lymphoblastic leukaemia	++	++	-
CEM-C7	Human acute T-cell lymphoblastic leukaemia	+	+	-
R-LICR LON/HMy 2	IgG-producing human myeloma	+++	+	ND
Peripheral blood	Human	(+)	(+)	(+)
Epithelial-like cells				
Hela	Human cervix carcinoma	+++	-	-
CV-1	Monkey kidney	+++	-	-
Fibroblast-like cells				
HFF	Human foreskin fibroblasts	+++	-	-
HE	Hamster embryonic cells	+++	-	-
LPV-HE ³⁸	Hamster embryonic cells, transformed by LPV	+++	++	+
F9	Mouse stem cells	++	-	ND
X63Ag8	Mouse plasmacytoma hybridoma	++	-	++
15E1	Mouse plasmacytoma hybridoma	++	-	++

The transfection procedures and CAT assays were done as described in Fig. 2b legend. After chromatography and autoradiography, the spots containing the acetylated (CM-Ac) and non-acetylated (CM) forms of ¹⁴C-chloramphenicol were cut out, placed in scintillation liquid and counted. The percentage of CM-Ac radioactivity in the total (CM-Ac + CM) was determined. -, No enhancement; +, 5-fold enhancement; ++, 5-20-fold enhancement; +++, >20-fold enhancement. The percentage of acetylated chloramphenicol in cells transfected with pA10-CAT2 (enhancer minus constructions, see Fig. 2a) was taken as the denominator and an enhancement factor was calculated for cells transfected with constructions carrying SV40, LPV and C μ enhancers, respectively. ND, not done.

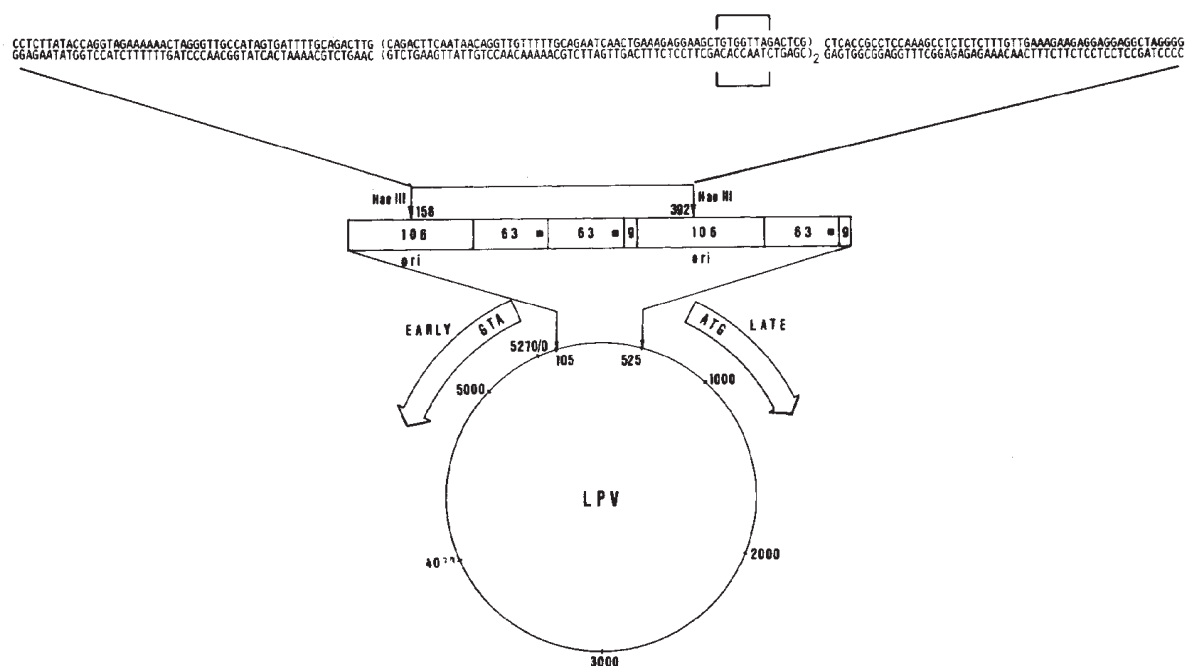


Fig. 1 Genomic organization of lymphotropic papovavirus (LPV). The early and late coding regions of LPV DNA are separated by a stretch of noncoding sequences of ~700 bp. This region contains several sets of direct repeats as represented by the open boxes (see text for further details). The numbers of base pairs which are homologous in each repeat are indicated by the numbers in the open boxes. A 234-bp *Hae*III fragment, containing the 63-bp repeat, was cloned into plasmid pA10-CAT2 (see Fig. 2). The sequences within the 63-bp repeat which resemble enhancer core nucleotides⁷ are indicated by solid squares. The complete nucleotide sequence²² of the *Hae*III fragment is given at the top, with the enhancer core nucleotides indicated by brackets.

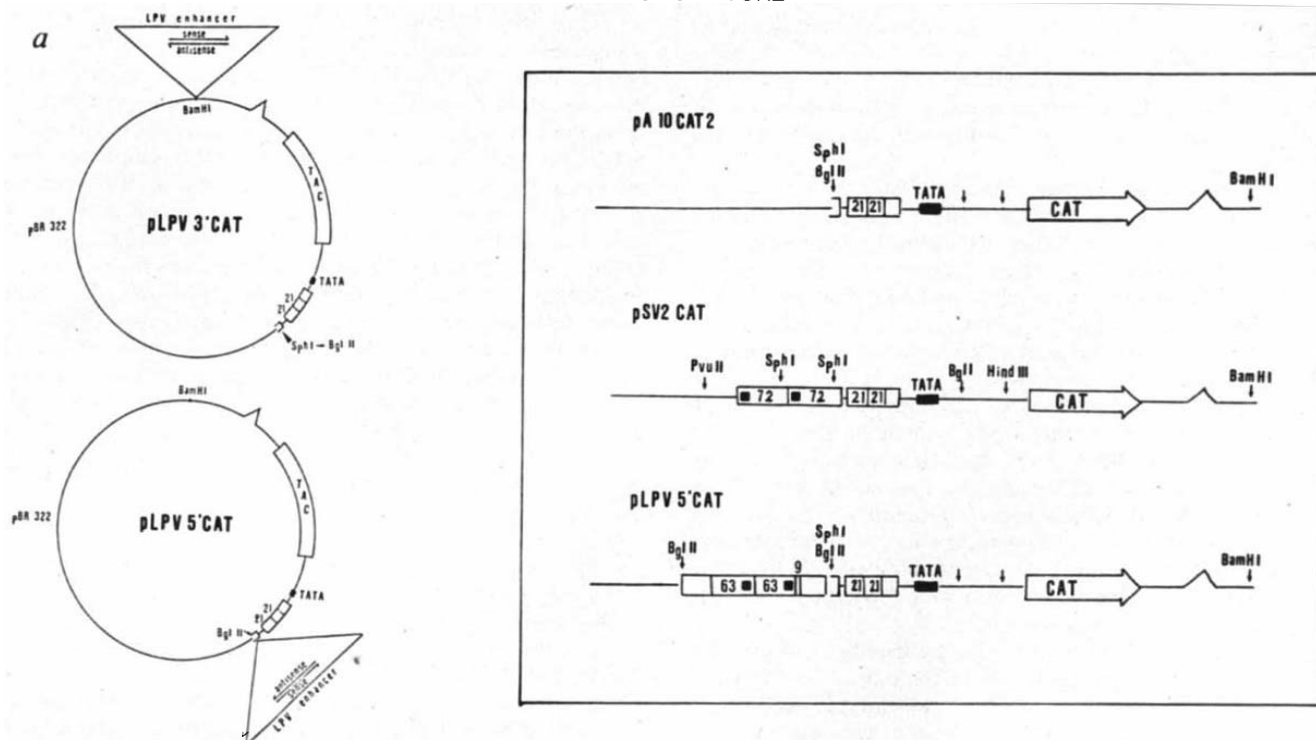


Fig. 2 *a*, Construction of pLPV-5'-CATs, pLPV-5'-CATa and pLPV-3'-CATs DNAs. Enhancer core nucleotides are indicated by closed boxes. *b*, CAT activity of BJA-B, MOLT-4 and HeLa cells after transfection with pLPV-CAT, pCμ-5'-CAT or pSV2-CAT DNA.

Methods. *a*, The 234-bp *Hae*III fragment (see Fig. 1) of LPV was excised, ligated to *Bgl*II linkers and cloned upstream of the CAT gene in the *Bgl*II site of pA10-CAT2 (ref. 9) in either orientation (sense or antisense). Another construction was made, containing the *Hae*III fragment downstream of the CAT gene in the *Bam*HI site of pA10-CAT2 (pLPV-3'-CATs). pA10-CAT2 and pSV2-CAT, which were also used for transfection experiments, have been described previously^{9,30}. *b*, BJA-B cells³⁵ and MOLT-4 cells³³ were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS. Cell transfection was carried out by the DEAE-dextran method^{33,34}. The cells (5×10^6) were washed twice with TBS¹⁴ and subsequently incubated at room temperature for 30 min with a mixture containing 10 μg of plasmid DNA in 50 μl TE (15 mM Tris, 1 mM EDTA), pH 7.8, 250 μl TBS and 300 μl DEAE-dextran (1 mg ml⁻¹). After incubation, the cells were washed again with TBS, resuspended in fresh medium, incubated at 37 °C, 5% CO₂ and 48 h after transfection, collected to determine CAT activity. The CAT assay was performed as described by Gorman *et al.*³⁰ with a few minor modifications. Standard enzyme reactions were performed with 100 μl of a total of 150 μl extract and were incubated for 90 min at 37 °C. The assay mixture final volume (171.5 μl) contained 0.2 μCi [¹⁴C]-chloramphenicol ($\sim 2 \times 10^5$ c.p.m.) and 4 mM acetyl-CoA in 0.25 M Tris-HCl buffer (pH 7.8). The reactions were stopped by extraction with 0.4 ml ethylacetate. The [¹⁴C]-chloramphenicol was dried and resuspended in 10 μl ethylacetate; its derivatives were separated by ascending TLC (0.25 mm silica gel) with a chloroform/methanol (95:5) solvent phase. The chromatography plates were then exposed to Kodak XAR-5 film for 12–24 h.

which can serve as an enhancer assay system as discussed previously⁹ (see Fig. 2a legend for details). Thus, activation of this transcriptional unit can be estimated through the production of both RNA and chloramphenicol-acetyl-transferase (CAT) activity, the amount of which is dependent on the enhancer inserted^{9,30}. The LPV fragment was cloned at the 5' end of the CAT gene into a *Bgl*II site in opposite orientations (pLPV-5'-CATs, pLPV-5'-CATa (Fig. 2a)). ('s' signifies sense, indicating that the orientation of the LPV fragment with the CAT gene is the same as it is with the early coding region of LPV; 'a' signifies the opposite, or antisense, orientation.) Similarly, the LPV fragment was inserted into a *Bam*HI site, located 3' of the SV40 polyadenylation signal (pLPV-3'-CATs). As a control, similar plasmids were constructed carrying the known enhancer of Cμ. The *Xba*I-E fragment of the mouse Cμ gene¹⁰ was excised and cloned in both orientations into an *Xba*I site of a test plasmid (pCAT-2 polylinker)^{31,32}. No difference in enhancer activity was

detected whether pA10-CAT2 or pCAT2-polylinker was used as a recipient for the same enhancer DNA fragments.

To test for the functional activities of the various plasmid constructions, purified DNA was introduced into eukaryotic cells using DEAE-dextran as facilitating agent^{33,34}. As LPV was adapted to grow lytically in the human B-lymphoma line BJA-B (ref. 35), these cells were chosen as recipients for the first series of transfection experiments. Little or no CAT activity was detected in BJA-B cells transfected with pA10-CAT2 (Fig. 2b), but when the 234-bp LPV fragment was inserted at the 5' end of the SV40 promoter region, the cells produced CAT, as demonstrated by the conversion of chloramphenicol (CM) into its acetylated forms (CM-Ac) (Fig. 2b). The conversion rate is independent of the orientation of the LPV fragment, as both the sense (pLPV-5'-CATs) and antisense (pLPV-5'-CATa) insertions gave similar conversion rates. Also, introduction of the 234-bp LPV fragment in the sense orientation at the 3' end of the polyadenyl-

ation signal produced activation of the transcriptional unit, thus fulfilling the criteria described for most other viral enhancer sequences. The relative level of enhancement at the 3' end (Fig. 2b; pLPV-3'-CATs), however, was three- to fourfold less than for the 5' construction. We are investigating the reason for this reduction.

The relative strength of the transcriptional activation by the LPV enhancer was estimated by performing a control experiment to measure the activity mediated by the well-characterized C_{μ} enhancer¹⁰. Insertion of the *Xba* I-E fragment at the 5' end of the SV40 promoter elements resulted in the production of CAT, independent of orientation (p C_{μ} -5'-CATa, s), at levels comparable with those of the respective LPV-containing plasmids in BJA-B cells (Fig. 2b).

To demonstrate that the CAT results directly reflect the CAT transcriptions present, we performed S_1 nuclease analysis of the CAT RNA to map the 5' ends. Although we found that the plasmid containing the LPV enhancer showed the same RNA start sites as the well-characterized plasmid pSV2-CAT that contains the SV40 enhancer (data not shown), the bands detected were very weak, preventing us from obtaining quantitative data. Therefore, the following experiments were carried out using the CAT assay, as this seemed more sensitive.

In investigating the host- and cell-type specificity of the LPV enhancer, because LPV seems to infect only cells of B-cell lineage, we used a human lymphoma cell line of T-cell origin (MOLT-4)³⁶ as a recipient for various plasmid constructions. Only the LPV 234-bp fragment and the SV40 enhancer^{9,22,30} activated CAT production in MOLT-4 cells, and no enzyme production was detected after transfection of the plasmid carrying the C_{μ} enhancer (p C_{μ} -5'-CATs) or the recipient plasmid without insert (pA10-CAT2) (Fig. 2b). A similar result was obtained after transfection of the plasmid constructions into two additional established human cell lines (JM-1, CEM-C7) with T-cell characteristics (Table 1). Thus, although the C_{μ} enhancer is active in cells of B-lymphoid origin, it does not seem to increase the transcription of transfected recombinant plasmids in the human T-cell lines used. On the other hand, the cell specificity of the LPV fragment seems less strict because LPV was also active in T cells and in several other non-B-non-T lymphoid cell lines (Table 1), whereas none of these cell types responded to the C_{μ} enhancer.

Because all the cell lines used in these studies have a transformed phenotype, it was essential to eliminate the possibility that transformation is required for LPV enhancer activity. We therefore transfected peripheral blood cells stimulated with concanavalin A and pokeweed mitogen and found that these cells recognize both LPV and C_{μ} enhancers (Table 1). Surprisingly, transfection of two mouse myeloma-derived cell lines, X63Ag8 (ref. 37) and 15E1 (B. Georg-Fries, personal communication), with pLPV-5'-CATs did not yield any CAT, although parallel transfections with p C_{μ} -5'-CATa showed marked CAT activity (Table 1).

To determine whether the activity of the LPV activator is restricted to haematopoietic cells, we analysed the enhancer activities after transfection of test plasmids into several established and primary cell lines (Table 1). Using the HeLa cell line, which is of epithelial origin, we found that, in transfection experiments the LPV enhancer (pLPV-5'-CATs) did not stimulate activity in these cells, whereas SV40 enhancer (pSV2-CAT) yielded large amounts of CAT (Fig. 2b, Table 1). A similar difference between LPV and SV40 enhancers was found in secondary human foreskin fibroblasts, CV-1 cells and primary hamster embryonic cells. To our surprise, the LPV-transformed³⁸ hamster embryonic cell line responded to both LPV and C_{μ} enhancers. It is unclear whether this results from a specific cell type, selected by transformation with LPV, or whether it results from the presence of a *trans*-acting factor³⁹ provided directly or indirectly by LPV T-antigen.

Our results indicate that a 234-bp fragment from the LPV genome contains an enhancer element. This selected fragment contains a 63-bp repeat harbouring the putative enhancer core

sequence⁷. Although probable, it remains to be shown that the transcriptional enhancement is mediated by this 63-bp repeat. Interestingly, this viral enhancer element exerts activity only in cells of human haematopoietic origin and in this respect differs from the mouse immunoglobulin heavy-chain gene enhancer¹⁰ which, in our hands, is active only in B-lymphoma cells. However, recent experiments by others indicate that the mouse C_{μ} enhancer is active in some T-cell lines of mouse origin (W. Schaffner and D. Baltimore, personal communication). This result could be explained by host-range differences and it is interesting that the LPV enhancer seems to show a host-range effect in addition to the cell-type specificity (Table 1). Preliminary evidence indicates that the inability of LPV to produce progeny in T lymphoblasts or other haematopoietic cells results from a lack of adsorption and/or uncoating of LPV particles (M.P., unpublished). Taken together, our data are compatible with the previous observation that enhancers interact with cellular molecules (refs 40-42 and P. Sassone-Corsi and P. Chambon, personal communication). However, only the presence of more than one class of cellular factors could explain our data satisfactorily. Our present research is directed towards the identification of such factors.

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A novel immune system against bacteriophage infection using complementary RNA (micRNA)

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The 'operon' theory of gene regulation¹, in which protein repressor molecules bind to the operator site of a gene to prevent its transcription, is now well established. Recently, however, cases have been discovered in which gene expression is regulated by complementary RNA molecules that are able to bind to the transcripts of particular genes and consequently prevent their translation²⁻⁵. For example, the synthesis of OmpF protein (a major outer membrane protein) in *Escherichia coli* is regulated by a short RNA complementary to a region of *ompF* RNA encompassing the 'Shine-Dalgarno' sequence⁶ and the translation initiation codon^{2,3}. This RNA has been termed micRNA (messenger-RNA-interfering complementary RNA), and its discovery has prompted us to construct an artificial micRNA system designed to regulate gene expression in *E. coli*⁷. A given target gene can be repressed by artificially producing an RNA (micRNA) complementary to the mRNA encoded by that gene. A micRNA system has also been used successfully in tissue-cultured mammalian cells^{8,9}. The use of artificial micRNAs to specifically regulate individual genes has great potential as a novel cellular immune system for blocking bacteriophage or virus infection. Here, we report that on induction of micRNAs directed against the coat protein and/or the replicase of the *E. coli* bacteriophage SP, phage proliferation was effectively prevented. We propose that the micRNA immune system provides an effective means of preventing viral infection as well as the expression of harmful genes in both prokaryotes and eukaryotes.

To design a micRNA immune system against phage infection in *E. coli*, we chose coliphage SP, a positive, single-stranded RNA, F-specific phage, which was isolated in Japan and classified in the group IV coliphages¹⁰. The entire phage genome consists of ~4.2 kilobases (kb) and its complementary DNA has been cloned (pSP219; Y.I. and A.H., in preparation). The nucleotide sequence of the entire genome reveals that the RNA phage codes for a maturation enzyme, coat protein, coat read-through protein and replicase (see Fig. 1a) (Y.I. and A.H., in preparation).

Previous work has shown that micRNAs that are able to hybridize with the target mRNA over the region containing the Shine-Dalgarno sequence and the initiation codon are the most effective repressors of gene expression⁷. Therefore, fragments A and B in Fig. 1 were used to construct the micRNA immune system: fragment A consists of the 247-base-pair (bp) *Cla*I/*Aha*III fragment encompassing the Shine-Dalgarno sequence and the initiation codon of coat protein; fragment B consists of the 159-bp *Rsa*I/*Aat*I fragment encompassing the Shine-Dalgarno sequence and the initiation codon of replicase. In addition to these two fragments, a DNA fragment (fragment C in Fig. 1) from the 3' end of the phage genome was also used; this fragment was derived from a *Bam*HI/*Pvu*I digestion of pSP219, a cDNA clone constructed from poly(A)-tailed phage RNA. Therefore, fragment C consists of ~690 bp: 411 bp from the C-terminal coding region of the replicase gene, 107 bp from the 3'-noncoding region, ~60 bp from the poly(A) tail, and 111 bp from the vector pBR322 (*Pvu*I-*Sca*I). These DNA fragments were inserted into the unique *Xba*I site of the micRNA cloning vector, pJDC406 (Fig. 1b), by blunt-end ligation. Thus, transcription of the inserted DNA is controlled by the strong *lpp* promoter and the *lac* promoter-operator, so that the production of a large amount of RNA transcript can be induced in the

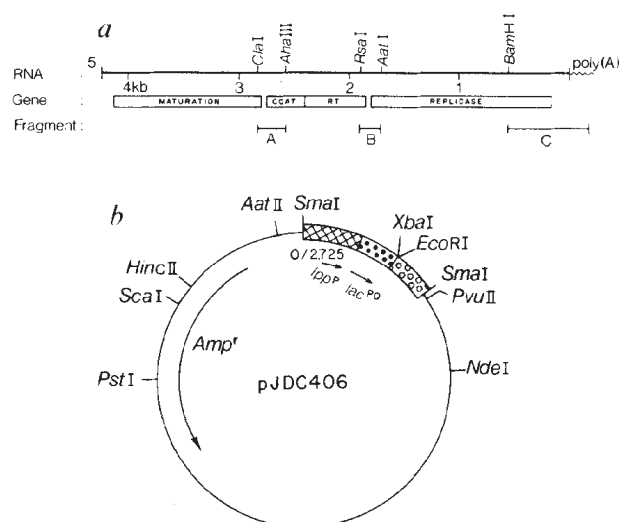
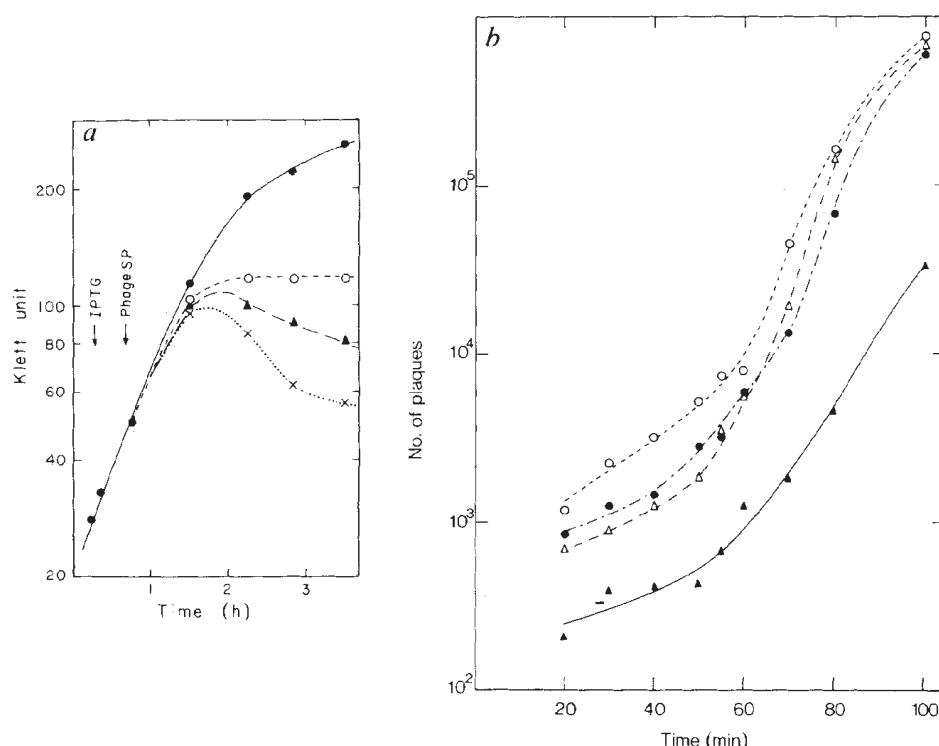


Fig. 1 a, Diagrammatic representation of bacteriophage SP. Numbers refer to the distance (in kilobases) from the 3' terminus. Only the relevant restriction sites are shown. RT, coat readthrough protein. Fragments A, B and C were used to construct the micRNA plasmids pMIC-A, pMIC-B and pMIC-C respectively. b, Restriction map of micRNA vector pJDC406. Numbers refer to size in kb. Cross-hatching represents the *lpp* promoter, filled circles represent the *lac*UV5 promoter-operator, and the open circles represent the *lpp* p-independent transcription terminator. pJDC406 was constructed from pJDC402 (for description see ref. 7) by deleting 0.3 kb downstream of the *lpp* terminator (including the downstream *Eco*RI site) and changing both *Hinf*I sites flanking the micRNA transcription unit to *Sma*I sites with the use of *Sma*I linkers (CCCGGG). The micRNA plasmids were constructed by inserting the fragment of interest into the unique *Xba*I site after creating flush ends with DNA polymerase I Klenow fragment. To construct pMIC-C:C, the smaller *Sma*I fragment from pMIC-C was inserted into the *Pvu*II site of a second pMIC-C, resulting in two micC genes on one plasmid. pMIC-A:B and pMIC-A:A were constructed by inserting the smaller *Sma*I fragment from pMIC-A into the unique *Nde*I site of pMIC-B and pMIC-A, respectively. pMIC-A:B:C was constructed by inserting the smaller *Sma*I fragment from pMIC-C into the unique *Aat*II site of pMIC-A:B.

presence of a *lac* inducer, such as isopropyl- β -D-thiogalactoside (IPTG)⁷. The orientation of the inserted DNA fragments was determined by restriction site analysis, and those plasmids in which fragments A, B and C were inserted in the anti-sense transcription direction with respect to the *lpp* and *lac* promoters were designated pMIC-A, pMIC-B and pMIC-C, respectively. As clear gene dosage effects have been demonstrated previously^{7,11}, we also constructed the following plasmids carrying two or three micRNA genes: pMIC-A:A (2 $\times$ micA, that is, two micA genes in one plasmid), pMIC-A:B (micA plus micB), pMIC-C:C (2 $\times$ micC) and pMIC-A:B:C (micA plus micB plus micC).

Figure 2a shows growth curves of *E. coli* JA221/F' *Lac*I^q (*hsdR leuB6 lacY thi recA Δ trpE5/F' lacI^q lac⁺ proAB*)¹² carrying the construct pMIC-A:B:C. micRNA production was induced by adding 2 mM IPTG before phage infection. Phage infection was carried out at a multiplicity of infection of ~15. Control cells carrying the vector plasmid alone were lysed after infection whereas cells carrying pMIC-A:B:C did not lyse in the presence of IPTG, although growth stopped after phage infection (Fig. 2a). This result indicates that the induction of micRNAs inhibited the proliferation of phage SP. Partial inhibition was observed even without the addition of IPTG, a result of incomplete repression of micRNA gene transcription in pJDC406 (ref. 7). Plaque formation was also inhibited when the cells carrying pMIC-A:B:C were used as indicator bacteria in the presence of IPTG. Figure 3a shows plaque formation of phage SP on *E. coli* JA221/F' *Lac*I^q carrying only the vector plasmid (pJDC406) in the absence and presence of IPTG. IPTG has little effect on either the number of plaques or the plaque morphology [Fig.

Fig. 2 a, Effect of phage SP on the growth curve of *E. coli* JA221/F'LacI^a containing pMIC-A:B:C or pJDC406. Cells were grown in L-broth¹³ containing 5 mM CaCl₂ in the presence or absence of the lac inducer IPTG (2 mM). ●—●, Turbidity of cultures containing pMIC-A:B:C or pJDC406 without phage SP in the presence or absence of IPTG. ×—×, Turbidity of a culture containing pJDC406 plus phage SP in the presence or absence of IPTG. ○—○, Turbidity of a culture containing pMIC-A:B:C in the presence of both IPTG and phage SP. ▲—▲, Density of a culture containing pMIC-A:B:C in the absence of IPTG and in the presence of phage SP. The labelled arrows indicate the times of addition of IPTG (to induce the micRNA genes) and of phage SP. **b**, Phage titre of the cell culture at various times after addition of phage SP. Circles represent *E. coli* JA221/F'LacI^a harbouring pJDC406; triangles represent JA221/F'LacI^a harbouring pMIC-A:B:C. Open symbols represent cultures grown in the absence of the inducer IPTG; solid symbols represent cultures grown in the presence of IPTG (2 mM) for 1 h before phage infection. Cells were grown in L-broth containing 5 mM CaCl₂ to a Klett-Summerson reading of 10, at which point IPTG was added to half of each culture. The cultures were grown for a further hour to a Klett reading of 55, at which time phage SP was added at a multiplicity of infection of 0.1. After 10 min the cultures were diluted 10-fold to decrease the changes of re-infection. At each time point, the appropriate dilutions of each culture were immediately mixed with a large excess of a stationary culture of *E. coli* A/λ and plated on to plates of L-broth. Plaques were counted after 12 h of growth. The initial amount of free phage was subtracted from each time point; this number was determined by titrating CHCl₃-resistant phage in the culture at 20 min. In all cases free phage numbered ~300.



3a(-) and (+)]. However, in the same *E. coli* strain carrying pMIC-A:B:C, the number of plaques was drastically decreased to about one-tenth of the control value (Fig. 3a) in the presence of IPTG. Note that 10 times more phage were plated out in Fig. 3b(+) than on all the others. In addition, the plaques in Fig. 3b(-) are much smaller than the control plaques. In the presence of IPTG [Fig. 3b(+)], the number of plaques decreased further, and the plaques were very small and difficult to see. These results are consistent with those shown in Fig. 2 and clearly demonstrate that micRNAs targeted against phage genes are able to effectively block formation of plaques and phage proliferation.

Figure 2b shows the phage titre of *E. coli* JA221/F'LacI^a carrying pMIC-A:B:C or the micRNA vector pJDC406 at various times after addition of phage SP. In the presence of IPTG, the number of infective centres decreased by about four-fold in cells carrying pMIC-A:B:C. At 100 min, the phage titre of the cells carrying pMIC-A:B:C in the presence of IPTG was

~0.05 of that observed for control cells (Fig. 2b). Note that *micA:B:C* had very little inhibitory effect in the absence of IPTG compared with cells containing the micRNA vector (pJDC406) alone. These results indicate that the inhibitory effect of *micA:B:C* is active throughout the phage infection. The poor yield of phage in the cells carrying pMIC-A:B:C in the presence of IPTG is probably a result of both direct inhibition of translation of the phage RNA and degradation of the phage RNA. Target mRNA degradation has been shown previously to occur after induction of micRNA^{3,7}.

To demonstrate that the initial effect of the micRNA is caused at least partially by the inhibition of phage protein production, *E. coli* JA221/F'LacI^a (containing either pMIC-A:B:C or the control plasmid pJDC406) infected with phage SP was labelled for 10 min with ³H-leucine. (Infection with phage SP was for 15 min at a multiplicity of infection of 5, after 1 h induction of the micRNAs with 2 mM IPTG.) In the presence of *micA:B:C*,

Table 1 Effects of individual and multiple micRNA genes on SP plaque formation

Plasmid	Absence of IPTG			Presence of IPTG		
	Titre*	% Inhibition†	Plaque morphology‡	Titre*	% Inhibition†	Plaque morphology‡
pJDC406	3,650	0	Normal	3,320	0	Normal
pMIC-A:B:C	2,120	42	Small	140	96	Very small
pMIC-A	2,510	31	Normal	1,290	61	Normal
pMIC-B	2,670	27	Normal	1,190	64	Small
pMIC-C	3,570	2	Normal	2,060	38	Normal
pMIC-A:A	1,860	49	Normal	310	91	Small
pMIC-A:B	1,710	53	Normal	300	91	Small
pMIC-C:C	3,170	13	Normal	1,510	55	Small

* This value is an average of at least two determinations (s.d. < 30%).

† Calculated relative to the values obtained with pJDC406.

‡ Plaque morphologies are shown in Fig. 3: a(+) = normal; b(-) = small; b(+) = very small.

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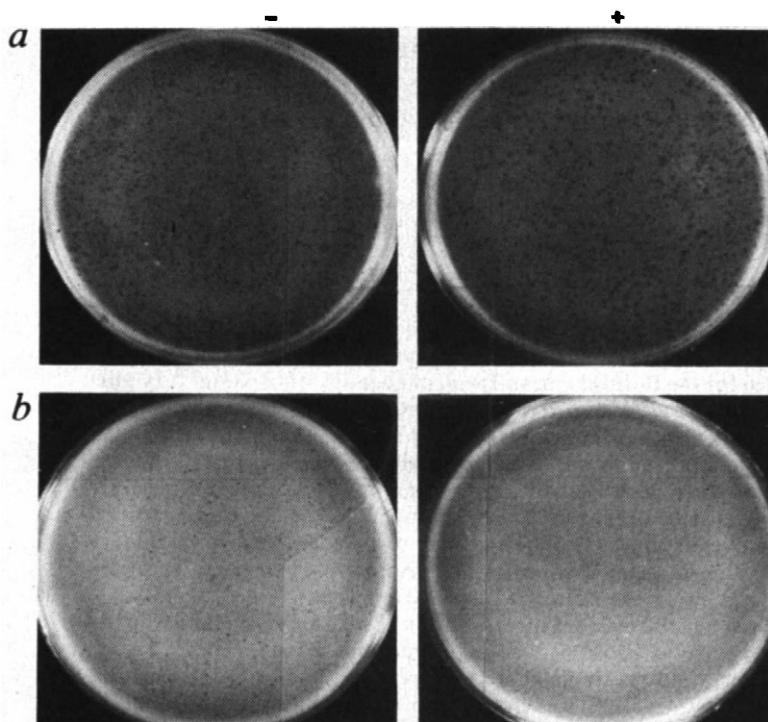


Fig. 3 SP plaque formation on a lawn of *E. coli* JA221/F'LacI^q containing pJDC406 (a) or pMIC-A:B:C (b) in the absence (–) or presence (+) of the micRNA gene inducer, IPTG. Cultures were grown to late log phase in L-broth¹³, at which time IPTG was added (to a final concentration of 2 mM) to half of each culture, and growth continued for an additional 2 h. IPTG was added to the warm L-broth top agar just before plating. Phage and bacteria were mixed 1 min before plating. The concentration of phage plated is the same in each case, except for plate b(+) in which 10 times more phage were used.

synthesis of the phage SP coat protein was greatly reduced compared with cells carrying only the micRNA vector pJDC406 (data not shown); other proteins were unaffected.

The inhibitory effects of pMIC-A:B:C were not due to its nonspecific effects on the cells. Cells carrying pMIC-A:B:C are fully sensitive to coliphages Q β (group III) and GA (group II), which are F-specific, positive, single-stranded RNA phages; the phage GA or Q β titre remained the same when these phage were plated, with or without IPTG, on *E. coli* JA221/F'LacI^q carrying either pMIC-A:B:C or pJDC406 (data not shown). This result indicates that such cells are fully capable hosts for F-specific phage infection, and that the effects of pMIC-A:B:C are specific to phage SP.

To determine which micRNA genes make the largest contribution to the immunity conferred by pMIC-A:B:C, phage titres were measured for *E. coli* JA221/F'LacI^q carrying the various micRNA plasmids (Table 1). In the case of the single micRNA genes, *micB* and *micA* appear to be more inhibitory to SP in the presence of IPTG than *micC*. Clear gene dosage effects are observed in the presence of two *micA* genes, and when the *micA* gene plus the *micB* gene are present. The *micC* gene in either the single or double configuration also shows inhibition of phage production although to a lesser extent than *micA* or *micB*. It should be noted that the *micC* RNA does not encompass the Shine-Dalgarno sequence or the initiation codon of replicase mRNA. This micRNA may prevent replicase from transcribing the positive RNA strand to produce the negative RNA strand, by forming a hybrid at the 3' end of the phage RNA.

We have demonstrated that micRNAs effectively block the proliferation of a specific virus. On the basis of our previous findings⁷, the micRNAs block the expression of specific SP phage genes by inhibiting the translation of these genes. It is important to note that the micRNA immune system is highly specific; the cells harbouring the micRNA genes directed against phage SP are resistant to phage SP, but not to phage GA or Q β . In particular, cells were not resistant to phage Q β despite its extensive homology to phage SP (Y.I. and A.H., in preparation). The high specificity of the micRNA immune system is important for its application in preventing viral infection and the expression of harmful or toxic genes in eukaryotic cells.

Combined with existing technology, the present report provides a promising prospect for the application of the micRNA immune system to both plant and animal cells. It is now feasible

to establish transgenic animals carrying a micRNA immune system(s) directed against a specific virus (such as poliovirus or foot-and-mouth disease virus, which are positive, single-stranded RNA viruses), by using micRNA genes that are stably integrated into the desired host genome. Similarly, micRNA immune systems directed against plant viruses can be introduced into the genomes of plant protoplasts which can subsequently be regenerated into mature fertile plants. Also, artificial micRNA repressors have great potential for use in the functional characterization of unknown genes, the repression of harmful genes such as oncogenes, and altered regulation of developmental genes.

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Note added in proof: Melton¹⁴ has recently shown that complementary RNA injected into frog oocytes will block translation of the specific RNA *in vivo*. In addition, Rosenberg *et al.*¹⁵ have recently shown that when RNA complementary to the *Krüppel* gene is injected into *Drosophila* embryos, a *Krüppel* phenocopy is produced. Furthermore, Izant and Weintraub¹⁶ have demonstrated that a micRNA gene can be inducibly expressed to function in mouse L-cells. Also, Pestha *et al.*¹⁷ have demonstrated in *E. coli* the production of β -galactosidase can be inhibited by the production of a complementary RNA.

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High-resolution structure of a DNA helix containing mismatched base pairs

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The concept of complementary base pairing, integral to the double-helical structure of DNA, provides an effective and elegant mechanism for the faithful transmission of genetic information^{1,2}. Implicit in this model, however, is the potential for incorporating non-complementary base pairs (mismatches) during replication or subsequently, for example, during genetic recombination^{3,4}. As such errors are usually damaging to the organism, they are generally detected and repaired. Occasionally, however, the propagation of erroneous copies of the genome confers a selective advantage, leading to genetic variation and evolutionary change. An understanding of the nature of base-pair mismatches at a molecular level, and the effect of incorporation of such errors on the secondary structure of DNA is thus of fundamental importance. We now report the first single-crystal X-ray analysis of a DNA fragment, d(GGGGCTCC), which contains two non-complementary G-T base pairs, and discuss the implications of the results for the *in vivo* recognition of base-pair mismatches.

Two principal schemes have been proposed for hydrogen bonding between non-complementary bases. One of these schemes² involves minor tautomeric forms of the bases (Fig. 1*a, b*); the resultant base pairs are stereochemically indistinguishable from complementary Watson-Crick base pairs (Fig. 1*c, d*). The estimated frequencies of minor tautomers correlate well with the observed frequencies of substitution mutations⁵ but there is, to date, no direct structural evidence for such base pairs. In the second scheme⁶, both bases are in the major tautomer form, but use different functional groups for hydrogen bonding than Watson-Crick base pairs (Fig. 1*e, f*). 'Wobble'

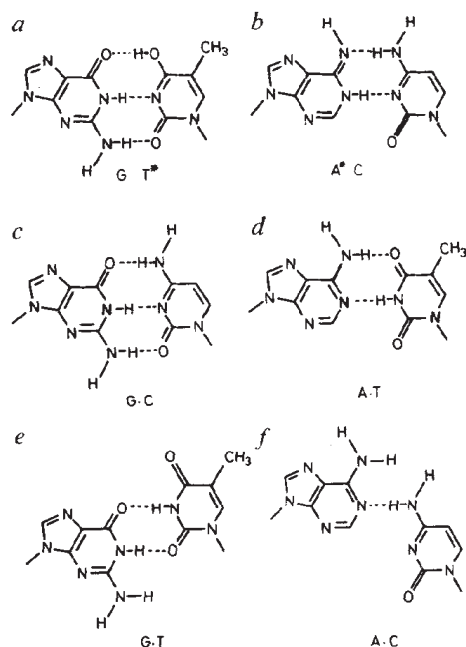


Fig. 1 Schematic diagram of various postulated base-pairing schemes. *a, b*, Tautomer pairs; *c, d*, Watson-Crick base pairs; *e, f*, wobble base pairs. Bases in their rare tautomer (imino/enol) forms are marked by asterisks.

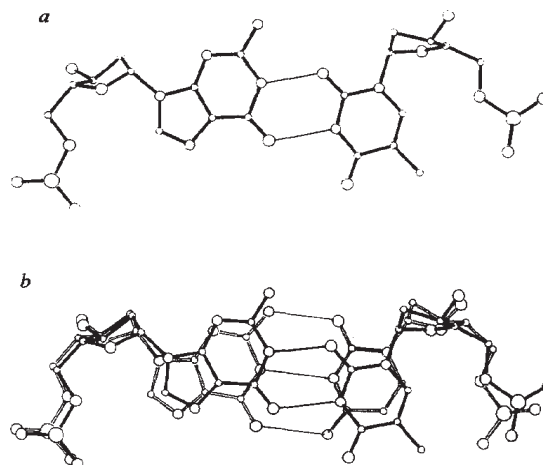


Fig. 2 *a*, The G-T wobble base pair observed in the crystal structure of d(GGGGCTCC). *b*, Superposition of the G-T wobble base pair observed in the crystal structure (dark bonds) on an ideal G-C pair. The figure was constructed by calculating the best molecular fit of the refined structure of d(GGGGCTCC) with a model of d(GGGGCCCC) derived from A-DNA fibre coordinates²⁴.

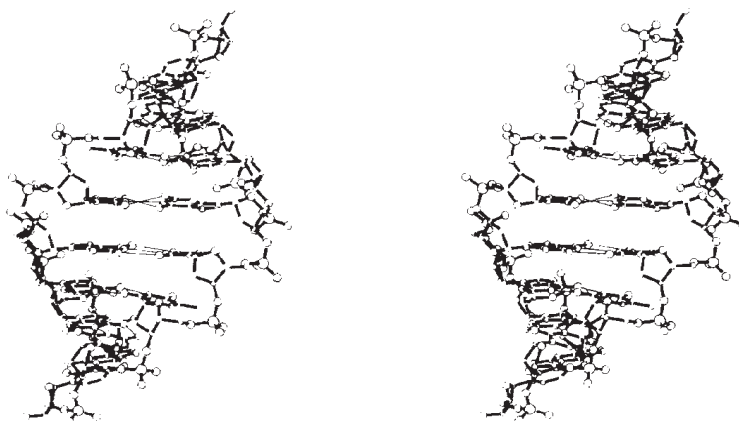
base pairs of this type have been observed in various transfer RNA crystal structures⁷⁻¹¹ and in recent nuclear magnetic resonance solution studies of deoxyoligonucleotides^{12,13} and polymers¹⁴. Mis-insertion of bases in their major tautomer form during replication is indicated by kinetic experiments with DNA polymerase I (ref. 15). The energetics of mismatched base pairs have been studied theoretically^{16,17}.

Detailed structural information about complementary base pairs is available from single-crystal X-ray diffraction studies of synthetic deoxyoligonucleotides¹⁸⁻²⁰. We have now extended this technique to mismatched base pairs by modifying the sequences synthesized so as to include non-complementary bases. The sequence d(GGGGCTCC) is a variant on two other A-DNA type octamers already investigated, d(GGTATACC)²¹ and d(GGGGCCCC)²². It was prepared by solid-phase triester methods²³ and crystallized from a buffered 3 mM solution of DNA (pH 6.5) with 28 mM MgCl₂, kept at 4 °C. The hexagonal crystals, space group P6₁, *Z*=1 octamer duplex, had cell dimensions of *a* = *b* = 45.20 Å, *c* = 42.97 Å, close to those of the parent octamers. Diffractometer measurements at 2 °C yielded 2,414 unique reflections.

A molecular model of d(GGGGCTCC) was constructed, using fibre coordinates for A-DNA²⁴. The G and T bases were related as in the minor tautomer pair of Fig. 1*a*. The coordinates of this model, after best molecular fit to d(GGGGCCCC), were the starting point for the refinement by constrained/restrained least-squares techniques. During the refinement no restraints were imposed on either the hydrogen bonding between the G and T bases or the sugar conformation. The present report describes the structure at the convergence of the CORELS²⁵ refinement at *R* = 19.4% for the 1,954 reflections with *F* > 2σ(*F*_o). Further refinement by the Hendrickson-Konert²⁶ technique reduced the *R* factor to 14.5% and allowed the localization of 56 water molecules, but did not result in any significant changes in the DNA structure. Full details will be reported elsewhere.

During the refinement the largest changes occurred at the positions of the G-T base pairs. The initial 2-fold molecular symmetry was, however, largely retained, as was the geometry of the six G-C base pairs. The bases in the mismatch pairs shifted substantially from their original positions, bringing the keto O-2 and the imino N-3 atoms of thymine within hydrogen-bonding distances of the imino N-1 and keto O-6 atoms of guanine. The refined structure shows unambiguously that both

Fig. 3 Stereoscopic view of d(GGGGCTCC) looking into the minor groove. The G·T wobble pairs lie on either side of the two central G·C pairs.



mismatches are of the wobble type with the bases in the major tautomeric forms (Fig. 2a), and gives the first view of a base-pair mismatch in the crystal structure of a DNA fragment. The geometry of the hydrogen bonds linking the bases is good with N...O distances of 2.81 Å and 2.85 Å in the first, and 2.50 Å and 2.66 Å in the second wobble pair.

Comparison of the G·T wobble pair with a Watson-Crick G·C base pair (Fig. 2b) indicates that the mismatch can be accommodated in the double helix without appreciable perturbation of the backbone. The sugar conformation remains C-3' *endo*. The C-1—C-1' distance is 10.2 Å, close to that observed in G·C pairs.

The greater part of the lateral displacement of the bases is the movement of the thymine into the major groove with root mean square (r.m.s.) displacements of 0.74 Å (for G) and 1.25 Å (for T) (Fig. 2b). As a result, the angles between the glycosidic bonds and the C-1'—C-1' vector, which are symmetrical in Watson-Crick pairs (54–58°)²⁷, assume values of 70° for T and 41° for G averaged over the two G·T pairs. The free keto O-4 atom of thymine now protrudes into the major groove and the N-2 amino group of guanine into the minor groove; these are available for hydrogen bonding to either solvent molecules or neighbouring double helices. The presence of the two G·T base pairs does not appreciably affect the overall conformation of the A-DNA helix (Fig. 3). The average helical parameters and torsion angles (Table 1) compare well with the corresponding values for the two isomorphous structures. The packing of the duplexes in the crystal structure is similar to that in other A-type structures¹⁸.

Stacking interactions between adjacent base pairs have a major influence on the conformational stability of double-stranded DNA, and have been shown to give rise to sequence-dependent conformational effects^{28,29}. Figure 4 compares the base-stacking overlaps in d(GGGGCTCC) with the equivalent steps of A-DNA from fibre data. The most striking change is

the improvement of the stacking at the wobble pairs on the 5' side of the thymine as a consequence of its displacement into the major groove (Fig. 4c). On the 3' side of the thymine there is virtually no overlap between the pyrimidines (Fig. 4b). This trend has also been observed for G·U base pairs in tRNA¹¹. The stacking of the purines on either side of the mismatch appears to be unaffected.

The structural results derived from the present investigation may have implications for the fidelity of replication and the enzymatic recognition of DNA containing a base pair mismatch. These results can be considered at four levels: the tautomeric form of the bases; local effects, such as base stacking in the neighbourhood of the mismatch; the influence of the base mispairing on the global conformation of the double helix; and the disposition of structural elements, particularly hydrogen donors and acceptors, in a mismatched base pair.

Fidelity of replication is maintained by an elaborate and as yet only partially understood mechanism involving specificity of base selection at the insertion step, proofreading (excision of incorrect bases) and post-replicative mismatch repair³⁰. Biochemical experiments show that non-complementary bases in their major tautomer forms can be incorporated during replication. Fersht and co-workers have found this to be the case for purine-pyrimidine mismatches by kinetic analysis of DNA polymerase I mis-insertion rates¹⁵. The wobble base pair found in the crystal structure examined here may be a good model for G·T mis-insertions.

There is good evidence that mis-insertion frequencies *in vitro* are dependent on the DNA sequence and that this may be a consequence of base stacking effects at the site of the mismatch³¹. The present investigation, based as it is on experimental data from a specific sequence, gives precise information about local changes in base stacking; it shows in particular an improvement in pyrimidine-pyrimidine stacking on the 5' side of the thymine base of the G·T wobble pair.

Table 1 Overall helical parameters and mean torsional angles for A-DNA structures

	d(GGGGCTCC)	d(GGGGCCCC)	d(GGTATACC)	Fibre model
Twist per b.p.	33.4°	31.6°	32.3°	32.7°
Base pairs per turn	10.8	11.4	11.1	11.0
Rise per b.p. (Å)	3.0	2.9	2.9	2.6
Base tilt	11°	13°	15°	19°
Propeller twist	15°	10°	13°	Variable
α	-81	-76	-62	-50
β	177	178	173	172
γ	65	62	52	41
δ	76	84	88	79
ϵ	-136	-156	-152	-146
ξ	-68	-70	-78	-78
χ	-163	-158	-160	-154

Helical parameters are as defined by Fratini *et al.*⁴¹. bp, Base pair. The glycosidic torsion angle is defined by the atoms O-1'—C-1'—N-1'—C-2 (pyrimidine) or O-1'—C-1'—N-9—C-4 (purine). Backbone torsional angles are defined by P- α -O-5'- β -C-5'- γ -C-4'- δ -C-3'- ϵ -O-3'- ξ -P.

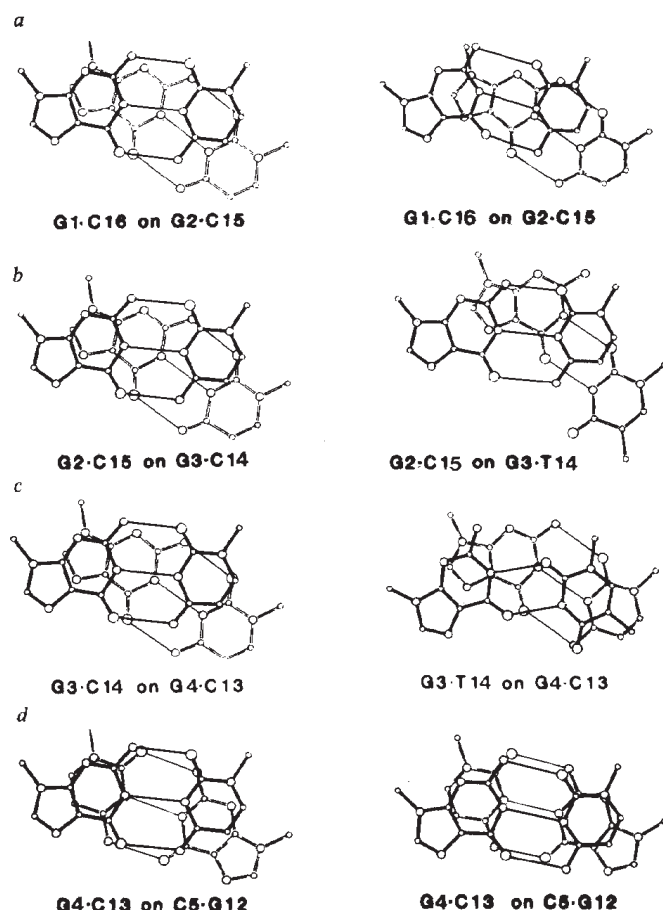


Fig. 4 Projected view of four steps of d(GGGGCTCC) (right) compared with equivalent steps derived from the A-DNA fibre model of poly(dG)·poly(dC) (left). The view is perpendicular to the mean plane through the upper base pairs and is in the 5' to 3' direction of strand (G1-G4). The upper and lower base pairs are differentiated by dark and light bonds, respectively. Only one half of the octamer is illustrated as the pattern of base stacking is symmetrical about the pseudo 2-fold axis. Note the similarity of the base stacking in the structures at the end step (a) and the centre step (d). At the site of the mismatch, the stacking of T on C is improved compared with C on C in the fibre (c) but the stacking of C on T (b) is unaffected.

Once the G·T wobble pair is incorporated in the double helix, it causes little perturbation to the global conformation. Nevertheless, it is discriminated against at various stages both during and after replication. The role of DNA polymerase in the recognition of incorrect base pairs is indicated by the variation of *in vitro* mutation frequencies with the nature of the polymerase³²⁻³⁵.

DNA polymerase is thought to have only one active site for synthesis, which is able to accommodate all four Watson-Crick base pairs³⁶. These base pairs have, as a characteristic structural feature, a pseudo 2-fold axis in the plane of the base pair perpendicular to the C-1'—C-1' vector, resulting in symmetrical disposition of the glycosidic bonds (Fig. 1c, d). As a consequence of this symmetry, the positions of the N-3 of guanine, the O-2 of cytosine, the N-3 of adenine, and the O-2 of thymine are approximately equivalent. These atoms are all potential hydrogen acceptors.

The X-ray analysis shows that G·T wobble base pairs in d(GGGGCTCC) lack diad symmetry. The guanine N-3 and thymine O-2 atoms are displaced from their positions in standard Watson-Crick base pairs and are no longer equivalent. The location of these functional groups may be an important factor in enzymatic recognition. The positions of other atoms in the

G·T wobble pair also change. The movement of the O-4 and the 5-methyl groups of thymine into the major groove may result in steric hindrance at the active site. Despite these differences, some major tautomer mismatches escape detection during *in vitro* DNA replication, possibly because of the lack of base specificity of DNA polymerase. However, at the post-replicative stage there may be a more specific recognition system with separate enzymes or active sites for the different mismatched pairs³⁷.

The present investigation gives high-resolution structural information about a G·T mismatch in a DNA sequence which crystallizes as an A-type helix. There is a need to examine base pair mismatches in all known DNA helices as there is accumulating evidence for conformational flexibility of *in vivo* stretches of DNA³⁸⁻⁴¹. We have recently solved, and are currently refining, other crystal structures and find that the G·T wobble pair, with the same hydrogen-bonding pattern, also occurs in a Z-DNA fragment d(TGCGCG) ($R=24\%$), in a B-DNA dodecamer d(CGCGAATTTGCG) ($R=23\%$), and in another A-type octamer, d(GGGGTCCC) ($R=15\%$). The results of these studies will be reported elsewhere. Our current investigations establish the feasibility of introducing errors into DNA sequences known to crystallize as A, B or Z helices, opening up the possibility of studying a variety of base pair mismatches and modified bases in different environments. This approach may lead to a better understanding, at the molecular level, of departures from complementary base pairing in the double helix.

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The future lies ahead

Gunther S. Stent

Solid Clues: Quantum Physics, Molecular Biology, and the Future of Science.

By Gerald Feinberg.

Simon & Schuster: 1985. Pp.287. \$17.95. To be published in Britain later this year by Heinemann.

IN THE Introduction to *Solid Clues*, Gerald Feinberg observes that 40 years ago no one predicted the enormous expansion of science then imminent. This unpredicted expansion included also the rise of "futurology", the very discipline dedicated to correcting our generally myopic view of the future. In contrast to old-time, apocalyptic or millenarian prophecy, which foretold the future on the basis of inspired visions in terms of value-laden, prescriptive proclamations, the predictions of futurology are phrased as rational arguments, based on some theory of the dynamics of history, and take the form of value-free and descriptive propositions.

Feinberg's avowed purpose is to predict the changes that will take place over the next few decades in the content of science and the lives of scientists. He concentrates his attention on physics and biology, the two branches of contemporary science which he finds to be progressing most rapidly and likely to produce the most important discoveries. Fortunately, as a professor of physics at Columbia University and having read some books and articles on contemporary biology, Feinberg is able to assess the present status of these two vanguard disciplines. Unfortunately, he seems to lack the grounding in the philosophies of science and history needed by the would-be futurologist, as well as an appreciation of what counts as a rational argument in the social sciences (rather than a mere assertion) and as a significant prediction (rather than a futurological platitude).

Feinberg will surprise few readers with his disclosure that one of "the main engines of change in science, the forces that act to change science from within and from the outside" is the development and application of new experimental, observational and mathematical techniques. Equally unsurprising is his revelation of the public attitude to science, reflected by the provision (or withholding) of financial support and the encouragement (or proscription) of certain topics of research, as another such main engine (or brake). So, how, being driven by these engines, is science going to advance in the future? Well, it will answer some old questions and discard others, with some of the answers to old questions in turn raising new questions. Is science going to progress indefinitely? Yes and no: "in some areas of science, new answers may bring us close to exhausting fun-

damental discoveries in the field . . . [whereas] other fields, such as cosmology, are more open-ended". Feinberg lists some of the currently unsolved problems of cosmology and suggests novel observational techniques whose development would open up entirely new cosmological vistas. But he does not present any explicit argument which shows why cosmology is open-ended.

For the future of biology, Feinberg seems safe in predicting that its "main work is to find answers to many of the open questions about living things". He believes that the origin of life, metazoan development — from zygote to cadaver — and the nervous system loom large among such questions. He predicts, moreover, that the incipient development of X-ray laser holography will advance the frontiers of biology as far as did the invention of the optical microscope in the seventeenth century. Whether there are any open-ended areas of biology, however, Feinberg does not say, nor does he trouble to justify his rather doubtful prediction that "biology will become more like the physical sciences, reasoning from general principles to specific cases".

Feinberg thinks that "of all the changes that have taken place in recent years in how science is done, none is more fundamental and sweeping than the use of computers . . . computers are now launching science into a new age". This phenomenon is attributable not merely to the capacity of computers to manage vast amounts of

data, such as those which accumulate in particle physics experiments carried out in high-energy accelerators or in molecular biological analyses of DNA and protein sequences. No, we are on our way to a new age because "one of the most intriguing possibilities is that computers will someday be developed that equal or even surpass human capabilities for creating new scientific ideas". Feinberg cites Herbert Simon's "Bacon" program of scientific induction as a first stage on the trip to the new age, apparently unaware that the inductive Baconian model of scientific discovery failed, not because, as he claims, "there was no straightforward method of finding pattern in the data", but because of the need to make an intuitive preselection of the kind of data in which a significant pattern is to be sought in the first place. (In the case of Simon's program, the computer is provided with such preselected data as paired values of force and acceleration and, finding that they vary in direct proportion, the computer "discovers" Newton's Law.) The well-known epistemological critique of claims on behalf of creatively "intelligent" machines notwithstanding, Feinberg imagines "a future in which in the early training of a scientist the computer will act as a teacher, and later as a research collaborator".

What about technological advances? Feinberg points to "nanoengineering", or direct physical manipulation of molecules or atoms, which will allow "the construction of objects of a previously unimagined complexity" and the production of novel materials whose strength exceeds that of presently available materials by factors of many thousands. And then there is, of course, the new "biotechnology", including genetic engineering, for which Feinberg is not alone in anticipating profound effects on the human condition, especially in so far as modifying the natural process of ageing is concerned.

Feinberg concludes his essay with a

New journals review

On 26 September *Nature* will publish the fifth annual review supplement devoted to science journals.

Criteria for inclusion of a journal in the 1985 issue are that:

(i) the first number appeared, or the journal was retitled, *between June 1983 and May 1984* (the second cut-off date allows at least three issues of a journal to have been published, the minimum number on which a reasonable judgement can be based);

(ii) it is published at least three times a year;

(iii) the main language used is English.

Publishers and learned societies are invited to send *four different issues of each suitable periodical, including the first and most recent numbers* (if from outside the United Kingdom, by air mail) to: The Review Editor, *Nature*, 4 Little Essex Street, London WC2R 3LF, England. Subscription details for both 1985 and 1986 should be included.



discussion of two philosophical issues. One is the place of science in the world; here, more prophet than futurologist, Feinberg strikes a righteous pose. He offers dogmatic pronouncements about a wide range of religious, ethical, political and sociological topics, which reflect mainly the unsophisticated views of the typical scientist-in-the-street. The other issue concerns the change in the nature of scientific explanation and the possible limits to understanding. Thanks to his professional background in modern physics, Feinberg has a much better grasp of this matter. He shows how the rise of the quantum theory has eroded some of the basic conceptual ingredients of explanation — foremost among them cause and effect. Moreover, many of the unsolved problems in both physics and biology pertain to highly complex systems in which tiny fluctuations in one variable have huge effects on other variables. The “chaotic” behaviour of such systems limits the degree to which we can hope to fathom them. Thus, as Feinberg points out to his regret, “future science may see more restrictions on what we are able to understand and predict”.

Also, because of “the immense expansion of scientific information . . . scientists can learn an ever smaller part of what is known and can concern themselves with an ever narrower segment of it . . . Science is thus in some danger of losing its character as a cumulative body of knowledge”. Despite his regret over these limits, Feinberg ends on an upbeat note, befitting his smiling, *simpatico* portrait shown on the dust jacket: “Present and future discoveries in science can transform human life. All of these represent bright prospects of future science”.

It may be mean to cavil at this well-meaning essay by a scientist whose interests and concerns clearly transcend his research specialization. But, all the same, it would be negligent for a reviewer not to point out that, just as in the “hard” natural sciences, there are also professional standards in the “soft” social sciences, and that, just as in particle physics, in futurology triviality is even more grievous than error. □

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At the interface

D.H. Everett

Adsorption and the Gibbs Surface Excess.

By D. K. Chattoraj and K. S. Birdi.
Plenum: 1985. Pp. 471.
\$71.40, £56.53.

IN THIS book Chattoraj and Birdi have set out to demonstrate the central role played by the Gibbs surface excess in describing adsorption phenomena in systems involving liquid–vapour, liquid–liquid, solid–vapour and solid–liquid interfaces, and in biological structures. This attempt to present a unified approach to a wide range of phenomena is to be welcomed. Unfortunately, the treatment leaves much to be desired.

The arguments employed often lack the rigour and self-consistency essential in discussions of thermodynamics. Thus, in the introductory chapter, the surface tension is introduced simply as “surface energy”, while in considering the equilibrium between bulk and surface phases the role of surface tension is ignored. Later, in Chapters 3 and 5, correct derivations of the equilibrium condition are given and the surface tension term is now included. In the earlier treatment the surface tension term is apparently hidden in the surface activity coefficient, as perhaps implied in the unhelpful comment that “the standard scale for the surface activity coefficient is uncertain”. Elsewhere different definitions of surface activity coefficients are introduced: that they are different quantities is suggested by the comment that

one must “use the appropriate reference scale”. Which? Also puzzling is this statement:

In the derivation of the Gibbs adsorption equation the contribution of the β -phase to the interfacial phase is implicitly neglected. The surface activity coefficients thus evaluated in *all probability* [my italics] include this contribution.

Words such as “in all probability” are unacceptable in a thermodynamic argument.

Description of the Gibbs method is, surprisingly, deferred until Chapter 3: it should have been in Chapter 1. The formal definition is correct, but its status is obscured by the comment that “this approach is extrathermodynamic but no mathematical error is involved in the postulate”. There is nothing extra-thermodynamic in Gibbs’s treatment: what it does is to provide a means of quantifying the macroscopic stoichiometric effect of the inhomogeneous distribution of matter in the interface. At no stage did Gibbs “imagine” that the “moles of solvent present in the interfacial region is zero”. His definition is independent of any molecular model, a feature which gives it its power, usefulness and wide applicability.

The book is illustrated by a substantial body of experimental material drawn from a variety of systems, much of it originating from the authors’ laboratories. Overall, one has considerable sympathy with Chattoraj and Birdi’s objectives, which are to some extent achieved. But it is unfortunate that to get the best out of the book the reader has to be constantly on the alert to avoid being misled. □

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Breeding resources

A.D. Bradshaw

Crop Genetic Resources: Conservation and Evaluation.

Edited by J.H.W. Holden and J.T. Williams.

George Allen & Unwin: 1984. Pp. 296.
Hbk £25, \$40; pbk £9.95, \$14.95.

THE key to this book is on p.212. In a well-documented review amongst a group of similar papers, J.R. Harlan assesses the value of wild relatives of crop plants in modern plant breeding. If anybody thought that we could do without all this wild material, here is the corrective — a sober account of the improvements in ecological tolerance, disease and pest resistance, quality, yield and even modes of reproduction which have come from relatives of present crop plants. But at the same time Harlan illustrates the consequent dilemma: because this potentially valuable material is scattered through innumerable species around the world, it is almost impossible to know where the next important source will be.

This is of course only half the story. The enormous losses of natural and semi-natural environments and the increasingly widespread use of new crop varieties mean that this wealth of genetic material is rapidly being depleted. Landmarks in the growth of concern were two FAO/IBP conferences (1968 and 1973) on the conservation of crop genetic resources. Also in 1973, the International Board for Plant Genetic Resources was formed to bring some international organization into the many efforts which were then starting, including the international network of regional centres for crop research and genetic conservation.

Ten years have now passed. In the book there are 24 papers reviewing the progress that has been made, each written by people who are eminent in their field. It is their undoubted experience which leaves a feeling of great unease about the magnitude of the task ahead.

It is easy to define some of the difficulties. In the first part of the book, on seed storage, Roberts and Ellis show elegantly the progress that has been made in quantifying deterioration by predictive equations, and Hanson how we are becoming able to store even some recalcitrant seeds. Ellis and Roberts discuss programmes for monitoring deterioration, advocating important new systems of sequential probability ratio tests. But while the International Rice Research Institute had 25,600 accessions in 1974, it now has more than 50,000. Despite improvements, the problem remains enormous.

The second group of papers deals with the capture and retention of variability. Gale and Lawrence consider how to overcome the problems of the decay of variability in conserved material, while

Chapman discusses the size of seed banks. This should be a crucial paper but few people will be convinced that the problem can be resolved on the basis that the variability in which plant breeders are interested is distributed at random; indeed several of the other contributions indicate that it is not, particularly because of the local origin of variation and the impact of local selection. The assessment by Singh and Williams is much more pragmatic.

Progress in *in vitro* preservation is covered by Withers; the problems of preservation of wild species *in situ* by various forms of nature reserves are assessed judiciously by Ingram and Williams; and there is a series of excellent papers on the evaluation of what has been collected. Apart from Harlan's review, we are left with the overwhelming picture that we can

conserve anything if we have the money, but that we are drowning in the twin problems of what to conserve and, once we have conserved it, how to use it. Papers on the fashionable new technique of some clonal variation and recombinant DNA suggest that this is where the future lies. I doubt it.

In his final paper, Holden argues that orthodoxy allied to critical commonsense is the real way forward. We shall need further collections, but now they must be only of threatened material and to cover particular deficiencies, and must be on an international basis: a very sensible end to a valuable and provocative book. □

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Mixed selection

Richard G. Klein

Hominid Evolution and Community Ecology: Prehistoric Human Adaptation in Biological Perspective.

Edited by Robert Foley.

Academic: 1984. Pp.296. \$37.50, £24.

WHILE the layman may be fascinated simply by the great age of ancient bones and artefacts, palaeoanthropologists value them mainly as data by which to follow the course of human biological and cultural evolution. Such objects are often ambiguous in their implications, and using them to construct a truly compelling and comprehensive story remains a difficult task. In large part, the problem is the actual quantity and quality of bones, artefacts and other real data, which are often distressingly sparse. In part, it is also a matter of the need for more powerful analytical frameworks, based largely on present processes that must also have operated in the past.

One framework that nearly all palaeoanthropologists use, at least implicitly, centres on the concept of natural selection and its ramifications, that is evolutionary theory. Robert Foley, the editor of this book, believes that palaeoanthropologists should apply evolutionary theory more explicitly, especially the idea of coevolution, according to which members of an ecological community evolve at least partly in response to evolutionary changes in other members. Thus, for example, as predators become fleet of foot, natural selection will tend to favour fleet prey, and fleet prey will in turn lead to selection for fleet predators.

To explore the implications of ecological thinking for palaeoanthropology, in 1981 Foley organized a symposium from which the present book grew. Six of the contributors attended the symposium and four did not, but each was asked to write a

chapter illustrating the role of ecological concepts in understanding human evolution. Perhaps not surprisingly, Foley's own contributions come closest to the mark. In the first of his two chapters, he succinctly outlines those aspects of evolutionary theory he thinks palaeoanthropologists will find most pertinent. In the second he discusses the variables — mode of locomotion, body size, population density, home range size, group size, diet, social organization, environment and so forth — that are clearly crucial for reconstructing the ecology of very early people. Unfortunately, most of the variables are very poorly controlled with real data, and Foley's ecological reconstruction is necessarily vague and inconclusive.

Among the other authors, perhaps the one who took Foley's brief most seriously was Garrard, who suggests that the extinction of some Pleistocene mammals in south-west Asia may have been caused by "human interspecific competition", by which he means competition between people and animals for the same resources. This is an interesting possibility, but Garrard presents no real supporting evidence, and his treatment of the broader issue of extinctions is very weak, despite its obvious relevance to the book's main purpose.

Two other contributors who deal reasonably closely with Foley's stated theme are Turner and Gamble. In an essay that is stimulating and thoughtful, but ultimately unconvincing, Turner argues that the Middle and Upper Pleistocene dispersal of human beings across Eurasia and into the Americas can be illuminated by an analysis of the dispersal of other large mammals, especially predators. In a stylistically similar essay dominated by highly readable argumentation rather than empirical demonstration, Gamble suggests that differences in available energy can be used to explain regional variation in the Palaeolithic archaeological record of Europe.

Some of the remaining chapters are closer to the book's theme than others, but

none deal with it directly. Potts concludes that early people probably accumulated most of the bones at some sites in Olduvai Bed I, though the sites need not have been "home bases" in the sense of modern hunter-gatherer camp sites. To support his conclusion, Potts cites the presence of numerous stone artefacts, as well as features of the bone assemblages that are probably due to people rather than to carnivores or other "natural" agencies.

Scott deals clearly and succinctly with the evidence for human occupation of Britain in the second half of the Last Glacial period. Conditions were obviously extremely harsh and, compared to the situation in many parts of western Europe, people appear to have been rare. Hill touches on the question of how we might distinguish bone accumulations created by hyenas from those created by people, but he focuses mainly on the scientific-philosophical nature of explanation or interpretation in palaeoanthropology. Although it might not have been Hill's intention, it could be concluded that he believes the interpretation of Plio-Pleistocene bone assemblages is something we can write about but not actually do.

Gowlett sees stone artefacts, humanly butchered animal bones and other archaeological traces as reflections of the existence of human mental abilities from at least two million years ago. He may also believe that natural selection sponsored progressive change through time in these abilities, but from his presentation one cannot be sure.

Finally, Roberts provides a clear, up-to-date and comprehensive overview of Pleistocene climatic change, particularly as it is known from deep-sea cores, and Stringer surveys the human fossil record, stressing broad issues such as the mode of human evolution (whether gradual or punctuated) and the possible importance of climate as a selective factor on body form in fossil people. As independent contributions in their respective fields, Roberts's and Stringer's chapters are among the best in the book, but they are also among the furthest from the central theme.

Sadly the book does not demonstrate the value of specific ecological concepts for understanding human evolution, if only because most of the authors fail to address the issue directly. Beyond lacking a common focus, the chapters also vary considerably in quality, and I think it can reasonably be asked whether they should have been published together. There is a great deal in the book to interest any palaeoanthropologist, but there is also much that would have been winnowed out or improved if the papers had been subjected to the refereeing process used by most specialist journals. □

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Recipes for success in the laboratory

Richard Perham

Methods in Molecular Biology, Vols 1 and 2.

Edited by John M. Walker.

Humana/Wiley: 1984/1985. Each volume pp.370, \$45, £41.

A CYNICAL older colleague once advised me that any successful academic would turn out to be one-armed, for only then would he be prevented from saying "On the one hand, this; on the other hand, that". By the same token, the author of a book on methods is confronted with a comparable problem; do I tell my readers about all the different ways to carry out this or that technique or do I limit myself to one which, from personal experience, I know works? Any practising molecular biologist will be aware that the same experimental technique is often carried out differently in different laboratories. The differences may be major, they may be minor: in some instances they will actually matter.

The contributors to these two volumes have, for the most part, opted for the second course. Each method selected for description receives an account replete with experimental detail, taken presumably from the author's laboratory notebooks. There are few caveats, even fewer descriptions of variations or alternatives. There are, of course, further references given for those interested in pursuing them and sometimes a range of useful hints on trouble-shooting. Volume 1 deals with proteins, Vol. 2 with nucleic acids. I cannot speak with authority on every method listed — the net has been cast very wide — but I could find no serious fault with the recipes for those methods I know well.

I do have some reservations. For example, Vol. 1 has 38 chapters covering techniques from the Lowry method of protein estimation to manual micro-sequencing, from polyacrylamide gel electrophoresis to hybridoma production. Some room might have been found for the chemical modification of proteins and the methods, enzymic and chemical, of peptide bond cleavage. Even something as basic as the S-carboxymethylation of proteins is tucked away in a description of peptide separation by hplc. On the other hand, automated sequencing is wisely eschewed, given the requirement for unusually sophisticated and expensive apparatus.

The second volume is, if anything, more comprehensive, the 53 chapters starting with DNA assay and isolation and ending with DNA sequencing. In between it covers mRNA, cell-free translation, plasmids, cloning in bacteriophage lambda and a host of other things. It was therefore disappointing to find, for example, no description of nucleic acid hybridization

directly in dried agarose gels or of the chemical synthesis of oligonucleotide probes.

But these are quibbles. Molecular biology now permeates so much of present research that a working knowledge of its methods can be essential; it is at once the queen and handmaiden of the biological sciences. The editor tells us that the intention was to collect descriptions of methods that would definitely work, particularly in the hands of novices, and I fancy that these books will rightly be welcomed in such quarters. They will not replace the fuller, more discursive treatments given by the originators of these techniques in, say, *Methods in*

Enzymology. Nor perhaps will they have the impact of Cold Spring Harbor's celebrated *Molecular Cloning: A Laboratory Manual* by Maniatis *et al.* (now sadly getting out of date) which informed so many beginners in their first fumbling steps in recombinant DNA work some three years ago. However, if, as intended, these books enable those of stout heart but limited experience to gain entry to the growing battery of molecular biological methods, they will have served a useful purpose. □

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Fields of physics

Graham G. Ross

Quantum Field Theory.

By F. Mandl and G. Shaw.

Wiley: 1984. Pp.354. Hbk £38, \$34.95; pbk £19, \$24.95.

CONSIDERING the importance of the subject, there has been a remarkable shortage of textbooks available for the student of field theory. For many years there were no significant texts to follow the pioneering, and excellent, accounts of Bjorken and Drell, and Schweber, and a very nice short book by Mandl. The situation has improved lately with the encyclopaedic work by Itzykson and Zuber, a very readable text by Ramond covering the path integral formalism and many others. It is in this context that Mandl and Shaw's *Quantum Field Theory* appears.

A pleasing feature of the book is that it is accessible to the new student, starting immediately with the Lagrangian formalism, and giving a detailed and careful treatment of quantum electrodynamics (QED). I liked the emphasis on performing explicit perturbative calculations and the implementation of the renormalization programme, although I was disappointed that the renormalization group is not discussed. Throughout, the text is augmented with worked examples and problems. It thus provides a welcome update of earlier textbooks on electrodynamics; it is more straightforward than Bjorken and Drell, more detailed than another recent introduction to the subject by Halzen and Martin, and should give both the experimental and theoretical student a good working knowledge of QED.

In its treatment of developments beyond QED the book has some shortcomings. There is no discussion of QCD, the non-Abelian gauge field theory for the strong interactions, and only a relatively short account of the gauge theory for the electroweak interactions. Indeed the concept of gauge invariance, central to modern developments in field theory, is only

developed in Chapter 12 and the path integral formalism, which simplifies the analysis of spontaneously broken theories, is not covered. As a result the discussion of the electroweak theory is disappointing, particularly as it does not present a calculation in a spontaneously broken theory beyond the leading order, nor cover the renormalization in such theories. This renders the book less useful than it might have been for it is in this area that much progress has been made which is not yet adequately covered for the novice graduate student.

Mandl and Shaw have produced a sound introduction to the subject of Abelian gauge field theories which should also be useful as a reference text for perturbative calculations. However it provides only part of a modern introductory course and should be read together with another text, for example that of Ramond, which deals with the advances beyond QED. □

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● After writing this review I received a second text on the same subject, *Quantum Field Theory* by Lewis H. Ryder (Cambridge University Press; £40, \$74.50). The similarity to Mandl and Shaw ends with the title, for Ryder provides an introduction to many of the recent advances in field theory, using the path integral formalism to derive Feynman rules for spontaneously broken non-Abelian gauge theories and including a discussion of some of the topological properties and solutions of these theories. The approach differs too, with emphasis on the more formal aspects, no problems and few worked examples. The book is quite readable and is a good introductory text suitable for a theory student, but is probably best read after Ramond's book. In many ways it complements Mandl and Shaw, although the lack of worked examples will be hindrance to the student wanting a full understanding of the subject, and again there is no explicit calculation presented for a spontaneously broken gauge theory. **G.G.R.**

Mathematics

Algebra, Geometry and Trigonometry in Science, Engineering and Mathematics. By M.V. SWEET. *Ellis Horwood*: 1984. Pp. 617. ISBN 0-85312-461-2. £14.50.

A Concise Introduction to the Theory of Numbers. By ALAN BAKER. *Cambridge University Press*: 1984. Pp. 95. Pbk ISBN 0-521-28654-9. Pbk £4.95, \$9.95.

Graph Theory and Combinatorics: A Volume in Honour of Paul Erdős. BÉLA BOLLOBÁS (ed.). *Academic*: 1984. Pp. 328. ISBN 0-12-111760-X. \$50, £32.50.

Inverse Semigroups. By MARIO PETRICH. *Wiley*: 1984. Pp. 674. ISBN 0-471-87545-7. £63.95.

Real and Functional Analysis, 2nd Edn. Part A, Real Analysis. By A. MUKHERJEA and K. POTHOFEN. *Plenum*: 1984. Pp. 364. ISBN 0-306-41557-7. Np.

Sets, Relations and Mappings. By T.S. BLYTH and E.F. ROBERTSON. *Cambridge University Press*: 1984. Pp. 97. Pbk ISBN 0-521-27285-8. Pbk £3.50.

A Survey of Trace Forms of Algebraic Number Fields. By P.E. CONNER and R. PERLIS. *Wiley*: 1984. Pp. 316. Pbk ISBN 9971-966-05-0. Pbk £17.65.

Theory and Applications of Linear Differential and Difference Equations: A Systems Approach in Engineering. By R.M. JOHNSON. *Halstead*: 1984. Pp. 183. ISBN 0-470-20106-1. £18.50, \$24.

Astronomy

Beyond the Black Hole: Stephen Hawking's Universe. By JOHN BOSLOUGH. *Collins*: 1985. Pp. 126. ISBN 0-00-217138-4. £7.95.

Comets: A Descriptive Catalog. By GARY W. KRONK. *Enslow*: 1984. Pp. 329. Pbk ISBN 0-89490-071-4. Pbk £15.50.

The Cosmic Cycle. By THEODORE P. SNOW. *Darwin Press, Box 2202, Princeton, NJ 08540*: 1984. Pp. 109. ISBN 0-87850-041-3. \$19.95.

Gamma Ray Astronomy. By RODNEY HILLIER. *Oxford University Press*: 1984. Pp. 202. ISBN 0-19-851451-4. £19.

Sternbilder: Blicke in den Nachthimmel. By ARGYRIS SFOUNTOURIS. *Ex Libris*: 1984. Pp. 111. Fr. 38.

Physics

Asymptotic Behaviour of Mass and Spacetime Geometry. FRANCIS J. FLAHERTY (ed.). *Springer-Verlag*: 1984. Pp. 213. Pbk ISBN 3-540-133351-8/0-387-133351-8. DM 30.

Atmospheric Electrodynamics. By HANS VOLLAND. *Springer-Verlag*: 1984. Pp. 205. ISBN 0-387-13510-3/3-540-13510-3. DM 98, \$35.70.

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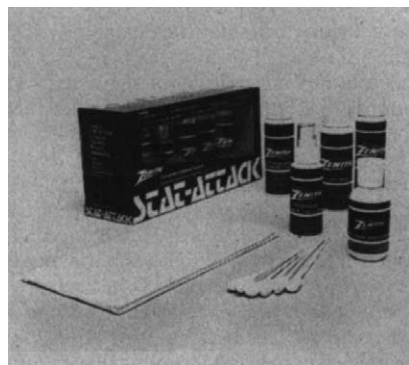
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● **BALANCE**, a new statistical software package from Elsevier Scientific, makes it possible to compare two series of measurements. **BALANCE** determines whether the means of two series of measurements are significantly different. The package can be used, for example, in comparing the results of two different medical treatments, investigating the effect of a drug, or of two analytical methods. This kind of comparison is probably the most frequently used statistical technique in industrial, clinical, chemical and quality control laboratories. **BALANCE** guides the operator automatically to the correct statistical test; it does not require knowledge of the statistical background by the user. **BALANCE** incorporates the following tests: tests for small numbers and for large numbers of observations; independent and related (paired) tests; one- and two-tailed (sided) tests; Student's *t*- and Cochran's tests (parametric tests); and Wilcoxon's matched-pairs signed-rank test and Mann-Whitney's *U*-test (non-parametric tests). *Reader Service No. 100.*



Stat-Attack, an anti-static computer cleaning system.

● **Zenith's Electronics' Service, Parts and Accessories Division** has introduced **Stat-Attack**, an anti-static computer cleaning system packaged in a convenient tray that doubles as a desk/shelf organizer. The **Stat-Attack** system contains an anti-static spray that protects equipment from static discharge. It can be sprayed directly onto printers and other static-generating computer equipment. When fitted with a special extension, it can be lightly sprayed onto carpets or countertops. It converts lint-free wipers (included in the system kit) into static neutralizing cloths. *Reader Service No. 101.*

These notes are based on information provided by the manufacturers. For further details circle the appropriate numbers on the Reader Service Card bound inside the journal.



A microcomputer, the **CompuPro 816/C** from **Viasyn Corporation of California**, is used to maintain a complete database on the behaviour and physical condition of **Ling-Ling, the Giant Panda in the US National Zoological Park**. This assists in determining her oestrous period, and hence the best time for breeding. (Picture courtesy of the Zoological Society of London.) *Reader Service No. 99.*

● The **IBM PC microcomputer** can now be used as a sophisticated data acquisition and control system for laboratory instruments using the **ADALAB-PC** from **Interactive Microware Inc.** A wide range of scientific instruments — including chromatographs, spectrometers, strip chart recorders, temperature controllers and *pH* meters — may be controlled by the **ADALAB-PC** using the specially designed **ADAPT** software which includes a menu-driven data acquisition program. The system provides accurately-timed collection of multiple data values; fast analog-to-digital (A/D) voltage sampling with direct memory access; data storage and retrieval from an extended RAM memory up to 640K; and a scrolling strip chart data display. A complete self-test checking procedure is also featured. **ADALAB-PC** consists of a single plug-in card, enabling the computer to interface easily with the lab instrument. The **IBM-compatible** system joins the established **ADALAB** products for use with the **Apple II micro**. *Reader Service No. 102.*

● **Packard Instrument** has developed an advanced network of gas chromatography instruments. A GC network of up to 12 satellite or conventional GCs and their accessories can be programmed and monitored from one central control station. The control station can be either the stand-alone **Model 830 network manager** or **Packard's top-of-the-line Model 439 GC**. The **Model 830** is a light and compact desktop unit. Control software for a number of GC accessories, including **Packard's One-Shot Samplers**, is built into the control stations. Four models of satellite GCs are available, covering the complete range of analytical requirements. *Reader Service No. 103.*

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● The **Oxymax-85 metabolic computer** from **Columbus Instruments** simultaneously measures: oxygen consumption, CO_2 production, respiratory quotient, respiration rate, relative tidal volume and temperature in one to four animal cages. The **IBM-PC personal computer** is used for data collection as well as a main system controller. An electronic laminar flowmeter is used at the input of each animal cage to ensure accurate measurement of air supply, and an electronic barometer, thermometer and hygrometer allow presentation of results in STP units, standardized to 760 mmHg, zero humidity and zero ($^{\circ}\text{C}$) temperature. Results of measurements are printed at 1-min intervals, and **Oxymax 85** is designed for 24-hour uninterrupted operation. *Reader Service No. 104.*

● A new option has been introduced for **ChemGraf**, a chemical modelling package based on the **DEC VAX minicomputer**. The new option is **ChemGuide**, an artificial intelligence program designed principally for novice and casual users of molecular modelling techniques. It helps the user to start using **ChemGraf**, select methodology, use specialist techniques, and tailor techniques to specific applications. Previously, only consultancy support could provide this assistance; support which is necessarily limited. Initially **ChemGuide** presents the user with a series of options. Depending on the option selected, **ChemGraf** commands are automatically executed, the user is asked for information, or is presented with a series of further options. **ChemGraf** systems, and the new **ChemGuide** program, are produced by **Chemical Design Ltd.** *Reader Service No. 105.*



Computer-controlled spectrophotometry from Beckman Instruments.

● Latest in the **Beckman Instruments, Inc.** range of software is **DATA CAPTURE**, which interfaces the **DU-50 series spectrophotometer** with the **IBM Personal Computer**. The software is part of a PC software library that interfaces a wide variety of **Beckman instruments** to personal computers. Through the direct integration of spectral measurement and processing capability, data can be acquired, analysed, displayed, archived and reported on a single economical computer. *Reader Service No. 106.*

● Epsilon Consultancy Ltd. is introducing a comprehensive software package to enable the Tablet Digitizer to be interfaced to a small computer so that the coordinate information produced by the Digitizer can be handled in a meaningful way. The true purpose of the software is to enable the user to make measurements of the material which has been digitized. The interface between digitizer and computer has been written to make use of the RS232 interface, because this interface is the most widely used one. By cabling the digitizer to the RS232 port in the computer, the user can start the software programs going and initiate a filing system by which the digitizations are stored in a manner which will make it easy, later on, to operate sorting methods on the files; calculations, tabulations and measurements can be done. The software can be expanded to handle special requirements, such as statistical comparisons between measurements.

Reader Service No. 107.

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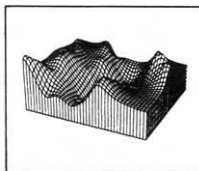
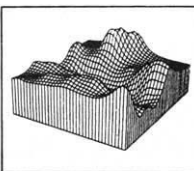
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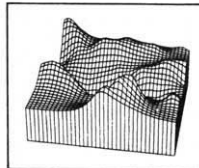
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● Two graphics packages designed specifically for scientists and engineers are now available on the IBM PC from Heyden Datasystems — Scientific Plotter-PC and Curve Fitter-PC. The Scientific Plotter-PC package plots graphs from data entered from disk, via the keyboard, or calculated by a user-defined equation. Axes are scaled according to the user's requirements, in either linear or logarithmic form, so that data are presented in the most suitable proportions. Plots may be superimposed in different colours; and there are 20 different plotting symbols in four size ranges to choose from. Variable-length error bars may be superimposed on the symbols. Curve Fitter-PC simplifies the fitting of a wide variety of curves to data which are input from disk or keyboard. The program automatically scales the plots and data, which may then be averaged and smoothed to reduce the number of points or average out background noise. Scientific Plotter-PC and Curve Fitter-PC are mutually compatible. Both packages are available in Apple II versions as well as the new versions on the IBM PC.

Reader Service No. 108.



Shown are examples of surfaces created with SURF and printed on a dot matrix printer. Figures show surface plot rotated 30 degrees (top, left), the same surface plot rotated 115 degrees (top, right), and this same surface plot rotated to 190 degrees (bottom, right).



Golden Graphics plotter output.

● Golden Graphics System is an integrated package that produces contour maps and three-dimensional surface plots, plus a variety of two-dimensional charts and graphs. Produced by Golden Software, Inc., the new package is designed to allow the user to visualize the implications of multivariate data. The Golden Graphics System consists of five applications: TOPO which creates contour maps; SURF which creates three-dimensional surface drawings, also known as block diagrams or fishnet plots; GRAFIT which creates xy graphs, bar charts, pie charts, line graphs, and scatter charts; QGRID which creates a regularly spaced grid from irregularly spaced data; and PLOTALL which creates plots from plotting commands that have been placed in a data file. The Golden Graphics System may be used with either a monochrome display card or a graphics card. If a graphics card is installed, output may be previewed on the screen. The Golden Graphics System runs on the IBM AT, IBM PC, and compatibles and requires 128K bytes of memory and one disk drive. Over forty different dot matrix printers are supported.

Reader Service No. 109.



Photomail, from Chorus Data Systems.

● PhotoMail, available from Chorus Data Systems, allows PC users to capture images with a standard video camera or VCR and transmit them to a remote PC via ordinary phone lines. Still-frame pictures are sent at a resolution of up to 640 × 400 × 16 colours or levels of grey. PhotoMail operates with IBM PC, XT, AT and compatible computers. PhotoMail is icon-driven and supports voice communications so users can converse while an image is being displayed. By using a 'mouse' or the keyboard, each party can simultaneously point to a specific area of the picture and have a cursor displayed on both screens. By drawing with the mouse, users can designate specific areas of interest on the display. Images can also be annotated with text to allow users to label areas of interest, type messages and create image descriptions or specifications.

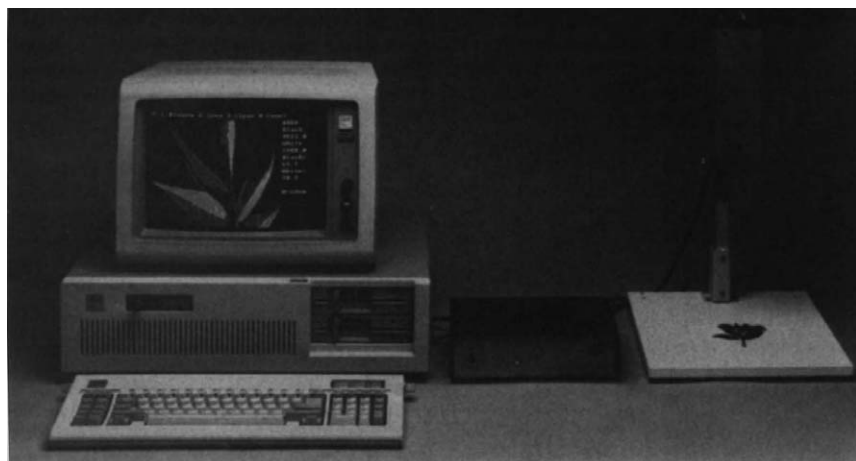
Reader Service No. 110.

● Walonick Associates has introduced a new version of the statistical analysis program StatPac. The new package (V5.2) incorporates enhancements suggested by hundreds of StatPac users. Owners of the package will receive this update at no cost. The new features in StatPac include banner crosstabs, probit regression, principal components analysis with multicollinearity diagnostics, and new graphics. Walonick Associates is a computer software publishing firm that specializes in vertical application software. In addition to StatPac, the company offers Forecast Plus, a graphics time-series analysis package, and Mach, a chemical-dependency assessment program.

Reader Service No. 111.

● The Microlink modular interface has been enhanced to provide a comprehensive hardware and software solution in data acquisition and control. With the addition of 15 new modules, Microlink, from Biodata, offers a choice of more than 40 modules including analog and digital inputs/outputs, transducer inputs, timers and counters. The new modules include programmable gain amplifiers, transient capture devices, pH and RTD inputs, frequency/event counters and alarms. With the availability of new software for use with the HP150 and BBC, Microlink hardware can now be used with most popular micros — including IBM PC, Apricot, HP 80 series, HP 9816, Sirius and Apple. Sample programs are provided in BASIC for each of these computers.

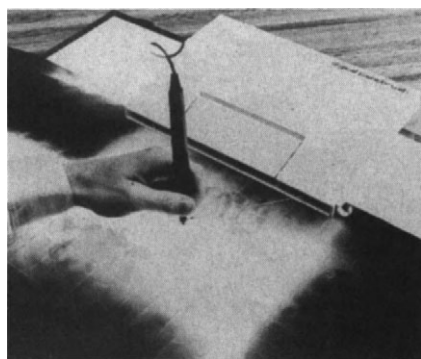
Reader Service No. 112.



Digithurst's MicroEye interface for graphics display.

● Digithurst announce the release of a version of MicroSight I which will produce images on the Tecmar Graphics Card. The images are read into the IBM via the Digithurst's MicroEye interface and are held in random access memory as a 256×256 pixel file at 255 grey levels. Using the Tecmar Graphics Card, it is possible to display 16 of these levels at any one time. Although the increase in the display quality will primarily benefit users in the graphics field, it is expected that the ability to display more grey scales will also be of benefit to those using Digithurst's MicroScale packages for computerized image analysis.

Reader Service No. 113.



Graphics imaging with the Digital Paintbrush System.

● The Computer Colorworks, a San Francisco-based company, has introduced an IBM PC version of its Digital Paintbrush System, a complete hardware/software colour graphics package designed for the manipulation of graphical data. A more powerful, advanced version of the already popular Apple product, the new system is designed for scientific and professional users of the IBM PC, XT, AT, and compatibles. Graphic images made with the Digital Paintbrush can be shown like slides on a colour monitor or TV screen, printed as hard copy or as transparencies for overhead projection, and photographed with various hood cameras and picture processors (including the Polaroid Palette) to make 35-mm slides.

Reader Service No. 114.

● ALLOCATE is a software package from the UK division of Elsevier, designed for the organizers of animal experiments or clinical trials. ALLOCATE rapidly assigns clinical or experimental subjects to treatment groups. The random allocation, on the basis of a chosen parameter such as body weight, is repeated (with statistical tests to assess uniformity of groups performed automatically after each iteration) until criteria set at the outset are met, or the 'best allocation so far' is accepted. Full details of groups and statistical results can be displayed on-screen, and printed out. Unequal group sizes are catered for, and an optional cull facility enables outliers to be deleted. Manual and disk are available for the IBM PC, DOS 2.0. ILEUM is a package for the accurate simulation of the effects of drugs on the *in vitro* guinea pig ileum. Drugs available for the ILEUM simulation include a dozen familiar agonists (which will contract the muscle) together with blockers of various sorts. Experiments to identify an 'unknown compound' can be carried out, for which the computer selects, at random, one of 20 'unknowns'. Random elements are incorporated into ILEUM to simulate the biological variability of response to the same dose of agonist. It is available for Apple II, IBM PC and BBC B computers.

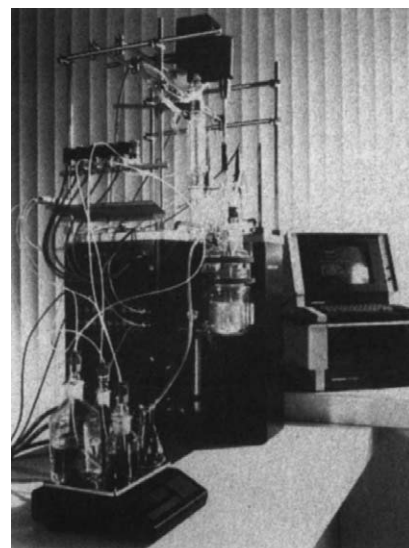
Reader Service No. 115.

● As an accessory to the Hewlett Packard 8451A UV/visible diode-array spectrophotometer, the new HP 89075A multi-cell transport automatically handles the concurrent measurement of up to seven samples over any length of time. The new transport provides accurate serial or random access to cells and is programmable, providing flexibility in method development. For applications such as kinetics, where multiple measurements must be taken and where data are acquired over long periods of time, the new product will deliver increased throughput and automated accuracy. The system mounts in the sample compartment of the HP 8451A spectrophotometer and uses standard 1-cm-square cuvettes and/or flow cells.

Reader Service No. 116.

● Northwest Analytical, Inc. has introduced a new statistical quality control charting package, called QUALITY ANALYST, which provides the software for both process capability studies and process monitoring. The package is available for the IBM and compatibles, MS-DOS computers, Burroughs B20, CP/M and Apple's Macintosh. QUALITY ANALYST provides an effective alternative to time-share and minicomputer-based quality control systems. QUALITY ANALYST is a complete package and can be used effectively as a stand-alone system. It can interface with other software packages and be used as the analytical core of a workstation using other software such as databases and spreadsheets. QUALITY ANALYST works well with standard communications packages and can process information transmitted from a mainframe database.

Reader Service No. 117.



Contalab, automatic laboratory reactor.

● Contalab, recently introduced by Contraves, is a computer-controlled automatic laboratory reactor that can operate for 24 hours a day, completely unsupervised. Complete reactions can be performed and monitored automatically and a simple question-and-answer dialogue guides the operator through all data entry procedures. The computer controls the experiment, compiles all the data, and produces a record of all results. The system automatically assigns alarm limits to enable 24-h operation. Contalab is especially suited to experiments which must be repeated many times or performed consecutively with minor changes (serial tests and optimization for example). Contalab consists of a reactor assembly with balance (for use in a laboratory fume cupboard), control unit with dual disk drive, peripheral equipment for data input and output, and software package (for controlling system operation and processing sequences). The reactor assembly is equipped as standard with a 1-litre reaction vessel, a reflux condenser and a stirrer.

Reader Service No. 118.

● Kratos Analytical's new DS 650 allows real-time data acquisition and system control for chromatography and data management. The DS 650 hardware is based on Data General's model 10/SP desk-top microcomputer and includes a 15 MB Winchester hard disk, one 368 KB diskette drive, 256 KB of main memory, a 12-inch green monochrome bit-mapped monitor, a graphics printer, and a multiplexed A/D converter. This converter is capable of accepting data simultaneously from up to eight detectors, using four separate times bases. The computer's menu-driven software guides the user.

Reader Service No. 119.

● Nelson Analytical Inc. of Cupertino, California, has developed an advanced chromatography workstation designed to meet the data acquisition, analysis and file management needs of individual chemists or small laboratories. The workstation comprises the Hewlett Packard 9000 series 200 computer, a Winchester disk drive and the HP ThinkJet printer, together with Nelson's XtraChrom software and one of four intelligent A/D interfaces. Up to ten instruments, each interfaced to up to two detectors, may be controlled by this HP3362A or B chromatography data system. As well as standard gas and liquid chromatography applications, the system can be used for capillary GC, ion chromatography and amino-acid analysis.

Reader Service No. 120.



● The Waters Expert multi-instrument chromatography software gives the Waters 840 data and chromatography control station simultaneous HPLC, GPC, GC and ion chromatography data reduction, together with facilities for multi-instrument control. Expert software provides chromatography data acquisition from up to four chromatographs simultaneously, with each system acquiring data from up to four detectors. In addition, up to four Waters HPLC systems can be controlled from the keyboard of the Waters 840 data and chromatography control station. The efficient multi-tasking operating system of the Waters 840 allows the monitoring of a separation from up to four different detectors simultaneously on the CRT screen. In addition, the Waters 840 provides virtually unlimited networking capabilities, including ETHERNET compatibility for high-speed data transfer. An IEEE, RS-232 interface allows communication with additional 840 systems or other computers.

Reader Service No. 121.

● The Consort II computer interface system, designed and developed by ICI (Australia), forms the basis of a comprehensive Apple computer-based data station for chromatography. The instrument provides a three-in-one system for accurate data collection on two channels, dual pump control for HPLC and control of peripheral devices, such as autosamplers and fraction collectors. Consort II operates with either an Apple II+ or IIe computer in conjunction with a comprehensive software system called Appligation II designed by Dynamic Solutions Corporation of California. The system can be configured by the user to cope with a wide range of chromatographic data including capillary GC, with sampling rates adjustable from 0.05 Hz up to 200 Hz. A special package for gel permeation chromatography is included. The Consort II computer interface system is marketed in the UK by Biotech Instruments.

Reader Service No. 122.

● Designed for use with the ACT Apricot range of microcomputers, Dynatech's new ABO/Rhesus blood-grouping programs interpret results from the microtitre plate, whilst offering flexibility of plate format, accumulative records of groups performed, and storage space for full data on up to 750 full plates (7,500 patients per data disk). The program also allows for alteration of interpretation parameters which may be used to re-interpret raw data from memory without the need to re-read the plate. This new program, developed in conjunction with Sanguin Computing, operates on the Apricot PC and Xi microcomputers and also on the Sirius microcomputer. Also new from Dynatech is its MicroBank System for the full automation of the entire blood-grouping operation in microtitre plates. Composed of three different work stations, the MicroBank has been designed to maintain patient integrity and avoid incorrect grouping due to operator error.

Reader Service No. 123.

● The latest version of LABTECH NOTEBOOK, a sophisticated menu-driven software program for real-time data acquisition, control and analysis, now supports hardware devices from Action Instruments, Cyborg, Datel, IBM, Interactive Microware, Keithley DAS, and Tecmar. Equipment from Acrosystems, Burr Brown, Data Translation, Metrabyte and Taurus is already supported by this software. NOTEBOOK delivers full hardware functionality of every data acquisition device it supports. The latest version, LABTECH NOTEBOOK 2.5, provides support for real-time thermocouple linearization and compensation. Data may be collected as temperature points directly from standard thermocouple devices, without requiring linearizing data conditioners. LABTECH NOTEBOOK supports the IBM AT, and it can also be integrated with spreadsheets like Lotus 1-2-3.

Reader Service No. 124.



Software-controlled colour measurement from Pye Unicam.

● The latest software-controlled colour measurement system from Pye Unicam is based on the PU 8800 high-performance UV/visible spectrophotometer. The new system uses an integrating spheroid and a fast, powerful software package for the HP 85 computer and is the only non-dedicated system that completely conforms to the requirements of the CIE, the governing body of colour science. The PU 8800 range includes a video display, built-in recorder and printer options and a choice of side or end-window photomultipliers. Available on tape and disk, the software provides measurements of colour or colour difference in up to three modes — diffuse or total reflectance and transmission. Illuminants A, C, D₆₅ and D₇₅ are offered, together with an additional two illuminants of the user's choice.

Reader Service No. 125.



Mettler software kit for thermal analysis.

● The performance of the Mettler FP800 thermal analysis system can be substantially enhanced by connection to a computer. For this purpose Mettler offers a software kit, which has been specially designed for the Epson HX20 hand-held computer. In addition to controlling the unit, it also permits various standard applications to be called up, results to be calculated and data stored for later processing. At very little extra cost, the FP800 can also be adapted for special applications, so that it operates automatically in accordance with national and international standards (such as melting point of plastics according to ASTM, clear melting point of fats according to AOCS). The program is written in MICROSOFT BASIC, so that it can be adapted easily to the individual requirements of the user.

Reader Service No. 126.